DISS. ETH NO. 23478

'The Contribution of PARP-1 and ELG1/ATAD5 to Genomic Stability'

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2016

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SUMMARY

DNA damage is a common threat to all cells, as it is a driver of malignant transformation. However, it can also be exploited in cancer therapy. Commonly used chemotherapeutics induce a high load of DNA lesions, which overwhelm the repair capacity of replicating tumor cells. Novel approaches aim at targeting backup DNA repair pathways in order to induce synthetic lethality in cancer cells that display specific defects in certain DNA repair enzymes. PARP inhibitors, such as Olaparib, are the prime example and have proven to be highly effective in the treatment of BRCA1- and BRCA2-deficient tumors. PARP1 is an enzyme involved in the repair of DNA single-strand breaks (SSBs). These small lesions may collapse into deleterious double-strand breaks (DSBs) when they collide with the replication machinery. In normal cells, DSBs are repaired in an accurate manner by the homologous recombination (HR) repair pathway, which relies on the activity of functional BRCA proteins. HR-deficient tumor cells die when treated with PARP inhibitors due to an accumulation of cytotoxic DSBs, which are then channeled into alternative, error-prone pathways, leading to genome rearrangements and ultimately apoptosis. PARP inhibitors are used as single-agent therapies, suggesting that the origin of lesions that rely on PARP activity must be endogenous. Intracellular reactive oxidative species (ROS) can lead to an accumulation of 8-oxo-2'-deoxyguanosine (G^o) in genomic DNA, which is potentially mutagenic due to its base-pairing properties with both cytosine (C) as well as adenine (A). G^o-containing lesions are repaired by the base-excision repair (BER) pathway, initiated by two different glycosylases: MYH addresses G^o:A pairs, removing the misincorporated A, while OGG1 excises the oxidized guanine (G^o) directly, but only from G^o:C base-pairs. Transient SSBs are generated during BER, which likely activate PARP that primes them for repair. Due to the abundance of endogenous oxidative DNA damage, we reasoned that their repair by BER leads to the generation of transient SSBs, which are the main contributors to the efficacy of PARP inhibitors in cells lacking active HR. Indeed, we show that MYH-depletion attenuates the sensitivity and genomic instability induced by Olaparib in HR-deficient cells. These results prove that processing of oxidative DNA lesions contributes to PARP inhibitor toxicity and therefore imply that tissue oxygenation and MYH status affect the efficacy of treatment.

An additional study in this thesis focused on another putative DNA repair factor, ATAD5, which has been proposed to be a suppressor of genome instability. Also we observed a hypersensitivity of ATAD5-deficient cells to certain DNA damaging drugs, such as the methylating agent MNNG, the interstrand crosslinking agent MMC and the PARP inhibitor Olaparib. Interestingly, ATAD5-deficiency causes retention of PCNA and ubiquitylated PCNA on chromatin, suggesting that ATAD5 is involved in their unloading from DNA. However, it is unclear at the moment if, and how, PCNA retention on chromatin leads to genome instability. The interaction with PCNA further suggests a role for ATAD5 in DNA replication, but we show here that this is in fact not the case. Instead, it might be required for post-replicative repair or other DNA repair processes, which remains to be investigated in the future. We have generated a useful set of tools to study the contribution of PCNA modifications on the genomic instability induced by ATAD5-deficiency, which will hopefully shed light onto important functions of both ATAD5 and PCNA in DNA metabolism.

ZUSAMMENFASSUNG

DNA Schäden sind gefährlich für alle Zellen, weil sie zu deren Entartung führen können. Gleichzeitig werden sie aber in der Krebstherapie genutzt. Häufig eingesetzte Chemotherapeutika induzieren eine grosse Anzahl von Schäden in der DNA, welche die Reparatur-Kapazität replizierender Zelle überschreiten. Neue Ansätze in der Onkologie sind darauf gezielt redundante Reparaturmechanismen zu inhibieren, um synthetische Letalität in Krebszellen zu induzieren, welche spezifische Defekte in Reparatur Enzymen vorweisen. PARP Inhibitoren, wie zum Beispiel Olaparib, sind ein Paradebeispiel hierfür und haben sich bereits als wirksame Behandlungsmethoden von BRCA1- und BRCA2-defizienten Tumoren bewiesen. PARP1 ist ein Enzym das an der Reparatur von DNA Einzelstrangbrüchen beteiligt ist. Diese kleinen DNA Schäden können zu folgeschweren Doppelstrangbrüchen führen, wenn sie mit der Replikationsmaschinerie kollidieren. Doppelstrangbrüche werden in normalen Zellen durch Homologe Rekombination akkurat repariert, welche von der Aktivität funktionsfähiger BRCA Proteine abhängt. Tumor Zellen mit defekter Homologen Rekombination sterben, wenn sie mit PARP Inhibitoren behandelt werden, aufgrund einer Anhäufung von toxischen Doppelstrangbrüchen. Diese werden schliesslich über alternative Mechanismen repariert, die allerdings fehlerhaft verlaufen, was zur Reorganisation des Genoms und letztendlich zum Zelltod führen kann. PARP Inhibitoren werden als Monotherapien eingesetzt, was darauf hindeutet, dass die ursprünglichen DNA Schäden endogener Natur sind. Intrazelluläre Sauerstoffradikale führen zur Akkumulation von 8-oxo-2'-deoxyguanosine (G^o) in genomischer DNA, was möglicherweise Mutationen verursacht, weil G^o mit Cytosin (C), sowie mit Adenin (A) Basenpaarungen eingehen kann. Läsionen, die G^o beinhalten, werden durch die Basen-Exzisions-Reparatur repariert, die durch zwei verschiedene Glykosylasen initiiert wird: MYH beseitigt das fehl-eingebaute A aus G^o:A Paaren; OGG1 hingegen entfernt das oxidierte Guanin (G^o) direkt, aber nur aus G^o:C Basenpaaren. Während der Basen-Exzisions-Reparatur werden vorübergehend Einzelstrangbrüche generiert, die wahrscheinlich PARP aktivieren, um sie für die Reparatur vorzubereiten. Aufgrund der Häufigkeit endogener, oxidativer DNA Schäden, folgerten wir, dass Einzelstrangbrüche, die während deren Reparatur vorübergehend entstehen, wesentlich zur Effizienz von PARP Inhibitoren in Zellen mit defekter Homologer Rekombination beitragen. Tatsächlich zeigen wir, dass MYH Knock-Down die Sensibilität und genomische Instabilität reduziert, welche durch Olaparib verursacht werden. Diese Resultate zeigen, dass die Reparatur oxidativer DNA Schäden zur Toxizität von PARP Inhibitoren beiträgt und deuten darauf hin, dass Gewebe-Oxygenierung sowie MYH Status die Wirksamkeit der Behandlung wesentlich beeinträchtigen.

Eine weitere Studie in dieser Arbeit konzentrierte sich auf einen weiteren mutmasslichen DNA Reparaturfaktor, ATAD5, welcher als Unterdrücker genomischer Instabilität beschrieben wurde. Auch wir haben eine Hypersensibilität ATAD5-defizienter Zellen gegenüber bestimmten DNA-schädigenden Substanzen, wie zum Beispiel MNNG, MMC und Olaparib, beobachtet. Interessanterweise verursacht ATAD5-Defizienz eine Akkumulation von PCNA und ubiquitiniertem PCNA auf Chromatin, was darauf hindeutet, dass ATAD5 an deren Abladung von der DNA beteiligt ist. Jedoch ist noch immer unklar ob, und wenn ja, wie, die Akkumulation von PCNA auf Chromatin zu genomischer Instabilität führt. Die Interaktion mit PCNA legt ausserdem

nahe, dass ATAD5 an der DNA Replikation mitwirkt. Wir zeigen jedoch hier, dass dies nicht der Fall ist. Stattdessen könnte es an Reparaturprozessen nach der Replikation oder anderen Reparaturmechanismen beteiligt sein. Wir haben nützliche Arbeitsmittel hergestellt, um die Beteiligung der PCNA Modifikationen, im Bezug auf die genomische Instabilität ATAD5-defizienter Zellen, zu untersuchen. Diese werden hoffentlich neue Einblicke in wichtige Funktionen von ATAD5 und PCNA im DNA Metabolismus liefern.

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ABBREVIATIONS

8-oxo-dGDP	8-oxo-2'-Deoxyguanosine-5'-Diphosphate
8-oxo-dGTP	8-oxo-2'-Deoxyguanosine-5'-Triphosphate
A^0	2-oxo-2'-Deoxyadenosine (2-oxo-dA)
ADPR	Adenosine Diphosphate Ribose
AMP	Adenosine Monophosphate
APE1	Apurinic/Apyrimidinic Endonuclease 1
AP-sites	Apurinic Sites
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related Protein
ATRIP	ATR Interacting Protein
BER	Base Excision Repair
BRCA1,2	Breast Cancer Type 1 Susceptibility Protein 1,2
CDC25	Cell Division Cycle Protein 25
CDK	Cyclin-Dependent Kinase
CHK1	Checkpoint Kinase 1
CHK2	Checkpoint Kinase 2
CKI	Cyclin-Dependent Kinase Inhibitor
DDR	DNA Damage Response
dNTP	2'-Deoxyribonucleotide
DSB	Double Strand Break
G^0	8-Oxo-2'-Deoxyguanosine (8-oxo-dG)
H2AX	Histon H2AX variant
HR	Homologous Recombination
LigI,III,IV	Ligase I, III, IV
MAP	MYH-associated Polyposis
MMR	Mismatch Repair
MTH1, 2	MutT-Homolog 1, 2
mtDNA	mitochondrial DNA
MUTYH/MYH	MutY Homolog
NAD^+	Nicotinamide Adenine Dinucleotide
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NO	Nitric Oxide
NUDIX	Nucleoside diphosphatase linked to another moiety, X
OGG1	Oxoguanine Glycosylase
PAR	Poly ADP-Ribose
PARP1	Poly (ADP-Ribose) Polymerase 1
PARPi	PARP inhibitor/inhibition
PARG	Poly (ADP-Ribose) Glycohydrolase
PCNA	Proliferating Cell Nuclear Antigen
RB	Retinoblastoma Protein
RNaseH2	Ribonuclease H2
RNKP	Polynucleotide Kinase-Phosphatase
ROS	Reactive Oxygen Species
R5'P	Ribose-5'-Phosphate
RPA	Replication Protein A
SOD1,2	Superoxide Dismutase 1,2
SSB	Single Strand Break
ssDNA	Single Strand DNA
TopI,II	Topoisomerase I, II

INTRODUCTION

CANCER & CANCER THERAPY

Cancer is a devastating disease, which is characterized by aberrant proliferation of cells due to genomic instability or, more specifically, by an accumulation of mutations favoring cell survival and growth. Cancer is the second leading cause of death in Europe and despite the tremendous efforts taken in the development of novel anti-cancer drugs surgery still remains the most effective treatment option. Radiation and chemotherapy are often combined treatment regimens after chirurgical removal of the tumor; however, the side effects induced by these therapies are detrimental due to their poor specificity.

To date, the most commonly used chemotherapeutics are agents that cause DNA damage, either directly or indirectly. The genomic instability induced by these drugs results in increased cell death, as cells are not able to cope with DNA damage exceeding a certain threshold. Cells possess elaborate DNA damage sensing mechanisms that signal to induce DNA repair or apoptosis if necessary in order to avoid malignant transformation and thus the onset of cancer. However, an urgent need to develop novel therapeutics to treat cancer arises from the poor specificity of the above-mentioned agents. More selective targets and drugs are required to minimize the side effects that many current anti-cancer therapies exert. Novel approaches aim at identifying synthetic lethal interactions to selectively target cancer cells while leaving normal cells unaffected [1]. Synthetic lethality is based on pre-existing mutations in cancer cells that render them dependent on a normally non-essential pathway. Exploiting the mutant phenotype of a cancer cell and targeting its synthetic lethal partner has already been proven to be a valuable approach in experimental setups [2-4], which have recently been approved for the treatment of a subset of tumors. Cancer cells deficient in certain DNA repair pathways are likely more vulnerable to further DNA damage, which can be induced either by increasing stress factors (such as oxidative or metabolic stress) or inhibiting redundant DNA repair pathways. The main challenges are now to identify novel synthetic lethal partners that are suitable for cancer therapy. Furthermore, it is crucial to overcome drug resistance, which arises from the high selection pressure and the adaptability of cancer cells due to their mutator phenotype.

Alterations in the sequence and organization of the genome range from small base modifications to gross chromosomal rearrangements and have been shown to accumulate over the course of neoplastic progression [5]. Mutations providing an advantage in growth and survival can cause uncontrolled cell division characteristic of malignant cells. Furthermore, mutations in oncogenes or tumor suppressor genes result in deregulation of cellular processes. This frequently leads to enhanced endogenous oxidative, metabolic and replicative stress, which in turn causes further DNA damage [6]. Adaptation to the stress phenotype is vital for cancer cells; however, it often results in dependency upon non-oncogenes to reduce the elevated stress levels and maintain balanced metabolism to ensure cell survival. Targeting these non-oncogene dependencies, which are required for cancer cell survival but dispensable in normal cells, may result in a synthetic lethal interaction [7, 8]. The elevated endogenous oxidative stress levels for instance, which arise from increased metabolic activity of cancer cells and oncogene activation, require adaptation to avoid adverse effects, such as oxidative damage of lipids, proteins and, most importantly, the DNA. Disturbing the cellular redox potential and reactive oxygen species

(ROS) balance has therefore been proposed to be a promising strategy for novel and more selective anti-cancer treatments due to the increased dependence of those cells on the ROS stress response [9]. Naturally, this includes protection from oxidative DNA injury by repair and prevention of this sort of damage.

CELL CYCLE

Proliferating cells, such as most cancer cells, have to enter the cell cycle, which enables division of the cell into two identical daughters. It is divided into different phases: In G1, the cells grow and prepare for replication, which is carried out in S-phase. Once 'quality control' of replicated DNA and chromatin assembly during G2 are completed, the cells are allowed to enter mitosis (M), where the duplicated chromosomes are separated into the two daughter cells. Most non-proliferating cells of the human body are in a dormant or quiescent state, G0, and need to receive appropriate growth-promoting signals to enter the cell cycle. Two hallmarks of cancer cells are their independence on external growth signals and their insensitivity to anti-growth signals [10, 11]. This is mainly due to frequent oncogene activation or tumor suppressor gene inactivation, which normally control cell cycle progression and ensure checkpoint activation.

Cell cycle regulation

Cell division is driven by cyclin-dependent kinases (CDK) that are only active, when bound to a non-catalytic, regulatory cyclin protein. Cyclin/CDK complexes orchestrate processes such as release of transcription factors, chromatin condensation and initiation of replication. The activity of the cyclin/CDK complexes is regulated by fluctuating cyclin expression levels throughout the cell cycle, CDK inhibitors (INK4 and CIP/KIP family members) that bind and inhibit cyclin/CDK complexes to ensure checkpoint control [12] and regulatory phosphorylations as well as dephosphorylations mediated by cell cycle kinases and CDC25 phosphatases, respectively [13]. These posttranslational modifications are required to control cell cycle progression and if necessary delay transition between the cell cycle phases. The tumor suppressor pRB mediates cell cycle arrest in response to anti-growth signals, while p53 enforces checkpoint activation upon cellular stress, through direct transcriptional induction of CKIs, including $p21^{Cip1}$.

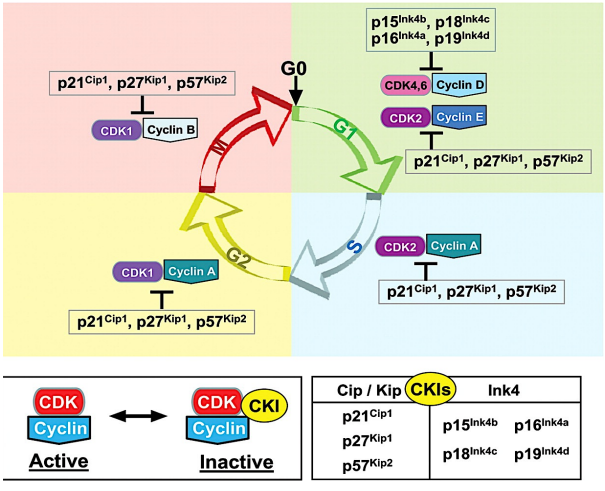


Fig.1: Cell cycle regulation by CDK/cyclin complexes and CKIs in mammalian cells. Different CDK/cyclin complexes in active state drive cell cycle progression and are inactivated by regulatory CDK inhibitors of the CIP/KIP and the INK4 families (from Fuster *et al.*, 2010 [14]).

REPLICATION

DNA is the carrier of the genetic information, encoding all proteins necessary for cellular metabolism and other functions of the cell. Unlike proteins, DNA exists only as a single non-replaceable copy in most cells (considering maternal and paternal DNA as distinct). It is therefore essential to ensure the integrity of the molecule and its faithful duplication during S-phase and transmission to the daughter cells. Replication thus has to be a highly accurate and controlled process. In cancer cells, replication is often deregulated and not restricted any more to a certain number of cell divisions. This limitless replicative potential was thus described as another hallmark of cancer [10, 11].

In eukaryotes replication is initiated at multiple replication origins, which are recognized by the hexameric origin recognition complex (ORC) [15]. Origin consensus sequences are unknown in Drosophila, Xenopus and human cells [16-18], but chromatin structure and histone acetylation seem to be important to initiate replication [19]. Licensing of origins takes place in late M- and G1-phases of the cell cycle through assembly of the pre-replication complex (preRC), consisting of ORC and the additional factors CDC6, CTD and MCM. In general, mammalian cells fire origins situated 30-150 kb apart [20] and weak or dormant origins may be activated to compensate for slow fork progression [21]. CDKs prevent re-replication by inhibiting origin licensing in S, G2, and early M phase [22].

Once origins have fired, replication has to proceed until the entire genome is fully replicated. Bidirectional unwinding of the origin region by the MCM helicase opens up a replication bubble with the two replication forks proceeding in opposite directions. Upon unwinding of the DNA, a ssDNA template is produced and immediately coated by RPA. Short 30 nucleotide RNA/DNA primers, termed Okazaki fragments, are synthesized by the primase activity of polymerase α (Polα). The replication factor complex (RFC) binds to primer-template junctions and loads the ring shaped proliferating cell nuclear antigen (PCNA) onto DNA. PCNA is an essential, homotrimeric sliding clamp that encircles the DNA and acts as a scaffold for a wide variety of factors involved in DNA metabolism [23]. Loading of PCNA onto the DNA during replication triggers a polymerase switch, leading to disassociation of Polα and replacement with processive polymerases Polδ or Polε [24, 25], which carry out the bulk DNA synthesis in a 5' to 3' direction. Posttranslational modification of PCNA by ubiquitin and SUMO expands the range of interaction partners and controls replication of damaged templates [26].

Replication of the leading strand is continuous, while the lagging strand synthesis occurs in a discontinuous fashion and is initiated at each Okazaki fragment. The replicative polymerases Polδ and Polε possess an intrinsic 3' to 5' exonuclease activity, which reduces the mutation frequency by about two orders of magnitude [27]. Additionally, mismatch repair (MMR) removes accidentally misincorporated nucleotides from the newly synthesized DNA strand immediately after replication and further reduces the mutation rate to 10^{-9} - 10^{-10} [28]. Continuous unwinding of the DNA creates topological problems of supercoiling and catenation. To release torsional strain in the molecule, topoisomerases introduce transient SSBs or DSBs at sites of ongoing replication [29].

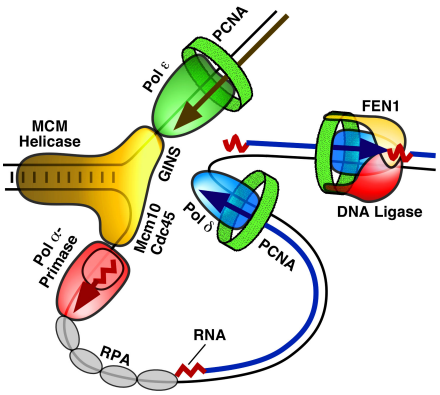


Fig.2: Eukaryotic DNA replication fork. The MCM helicase unwinds the DNA strands, which are then protected by the single-strand binding protein RPA. The leading strand is replicated in a continuous manner by DNA polymerase ε with the help of its processivity factor PCNA. The lagging strand is replicated in shorter fragments, termed Okazaki fragments, each beginning with a RNA/DNA primer made by the primase and subsequently extended by DNA polymerase δ. Okazaki fragments are ligated by the FEN1/DNA ligase complex (modified from Garg and Burgers, 2005 [30]).

PCNA

PCNA is a homotrimeric ring that encircles the double stranded DNA and plays a key role in replication and repair. It serves as a processivity factor for the replicative polymerases and is also a moving platform for other interacting proteins during DNA replication, repair and chromatin remodeling. Most of these proteins interact with PCNA via their PCNA-interacting peptide (PIP) or -motif [23]. Posttranslational modifications of PCNA during normal replication or in response to DNA damage regulate its interaction with other proteins and channel it into different repair pathways.

Mono-ubiquitylation of PCNA at lysine 164 in response to replication fork stalling at DNA lesions is carried out by the E2 ubiquitin-conjugating enzyme Rad6 together with the E3 ubiquitin ligase Rad18 [31] and activates the DNA damage tolerance pathway (DDR). Here, specialized translesion synthesis (TLS) polymerases, which bind to mono-ubiquitylated PCNA, are able to bypass the lesion due to damage-adapted active sites, reduced specificity and their ability to extend DNA synthesis from lesions. However, this mechanism is often error-prone, especially on undamaged templates, due to the reduced fidelity of these polymerases and the lack of an exonuclease proofreading activity [28]. A more detailed description of the TLS mechanism will be discussed in the chapter 'DNA damage bypass' of this thesis.

PCNA can also be poly-ubiquitylated on lysine K164 by the E2 pair Ubc13 and Mms2, together with the E3 ligase Rad5 [32]. This modification coordinates an error-free repair pathway, which is still not very well characterized but probably involves the presence of the sister chromatid [33].

In addition, PCNA can be SUMOylated during S-phase or upon high doses of DNA damage (*e.g.* MMS). This modification is mediated by the SUMO-specific E2 Ubc9 together with the E3 Siz1, primarily on K164 and to a lesser extent on K127 [31]. PCNA SUMOylation affects pathway choice, preventing homologous recombination in favor of ubiquitin-dependent lesion bypass [34]. The helicase Srs2 (or its human analog PARI) is recruited to $PCNA^{SUMO}$ via its PIP and SIM domains. Once recruited to stalled replication forks, Srs2 inhibits recombination by using its translocase activity to displace Rad51 from ssDNA, preventing strand invasion and homology search [35, 36]. Srs2 and PARI are thus often referred to as 'anti-recombinases'. Interestingly, it was recently shown that SUMOylation frequently targets entire groups of physically interacting proteins that bear SUMO-interacting motifs (SIMs) [37]. This protein-group SUMOylation may

act as a 'glue' to promote and strengthen intramolecular interactions of functionally engaged protein fractions, *e.g.* in nuclear assemblies, such as DNA repair foci [38].

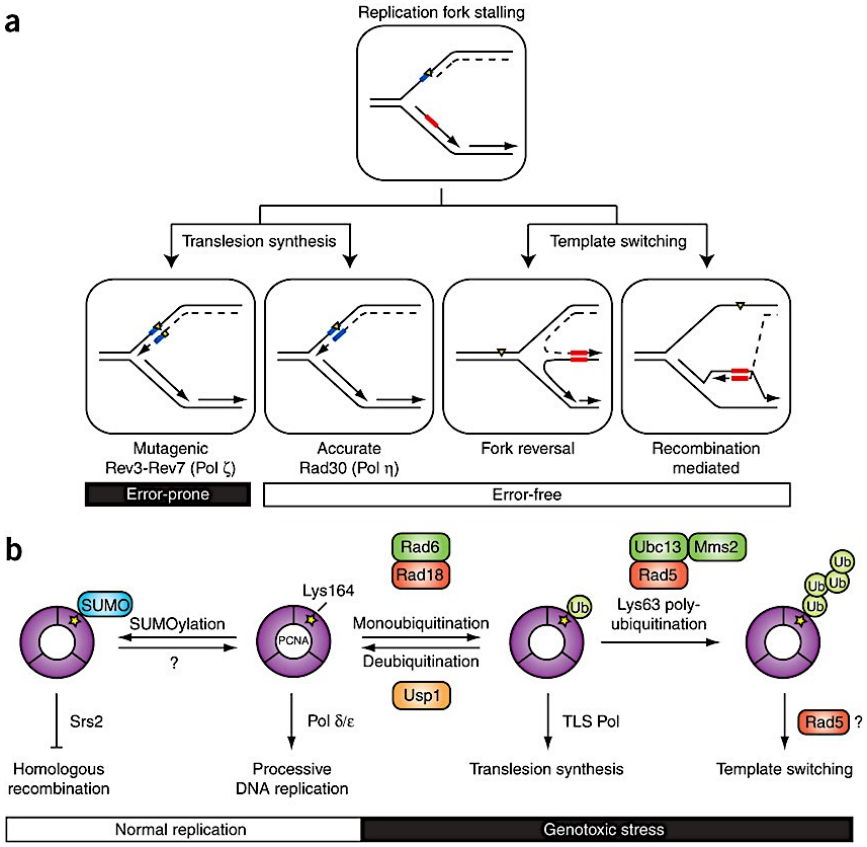


Fig.3: Replication Fork Stalling and its consequences. a) DNA lesions (yellow triangles) may block processive DNA replication (dashed lines). DNA damage tolerance mechanisms allow bypass of such DNA damage by translesion synthesis (TLS) or using the undamaged sister chromatid as a template (template switching), which involves structural rearrangements of the replication fork. Fork reversal includes the formation of a four-way junction ('chicken foot'), while recombination-mediated template switching involves D-loop formation. **b**) Post-translational modifications of PCNA and enzymes that carry out the modifications. During S-phase, *S. cerevisiae* PCNA is SUMOylated at Lys164 (yellow star), which recruits the helicase Srs2 to inhibit homologous recombination during normal replication. Although SUMOylation is reversible, deSUMOylation of PCNA has not been documented thus far. Upon induction of DNA damage, PCNA is ubiquitinated at Lys164. Monoubiquitinated PCNA facilitates recruitment of TLS polymerases and therefore lesion bypass, while Lys63-linked polyubiquitylation of PCNA is associated with template switching. Rad6 and Ubc13-Mms2 are E2 ubiquitin-conjugating enzymes, while Rad18 and Rad5 are E3 ubiquitin ligases. Usp1 together with Uaf1 is a deubiquitinating enzyme (from Chang and Cimprich, 2009 [39]).

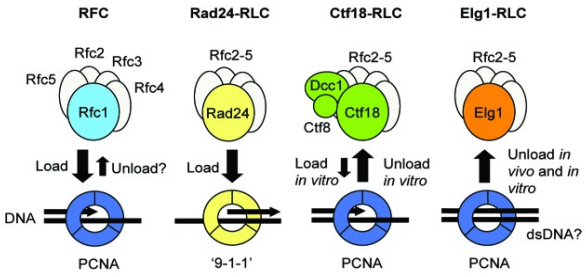
Replication factor C complexes

Loading of PCNA onto the DNA at the 3' end of a primer template junction or onto nicked DNA is promoted by the canonical replication factor C (RFC) complex. RFC is an essential hetero-pentamer, which is comprised of five AAA+ ATPase subunits: one large subunit, RFC1, and four small subunits RFC2-5. Together they mediate ATP-dependent binding to PCNA, ring opening, insertion of suitable DNA and subsequent ring closing [40, 41]. Upon ATP hydrolysis, RFC ejects PCNA, leaving it on the DNA in closed form [42, 43]. PCNA loading is required at each Okazaki fragment, it thus seems reasonable to assume that continuous recycling of PCNA is necessary for

sustained replication of the lagging strand. Human RFC also has PCNA unloading activity *in vitro*, however, the efficiency as a PCNA loader far outweighs unloading efficiency [44].

In addition to the canonical RFC, three RFC-like complexes (RLCs) have been identified, which display alternative functions during DNA replication and repair. These RLCs are composed of the small RFC subunits RFC2-5 together with an alternative large subunit replacing RFC1: RAD17 (Rad24 in budding yeast), CTF18 and ATAD5 (Elg1 in budding yeast). RAD17-RLC loads the Rad9-Hus1-Rad1 (9-1-1) complex onto DNA and thus induces an ATR-mediated DNA damage response (DDR) [45]. CTF18-RLC, together with its two additional subunits Dcc1 and Ctf8, interacts with PCNA and is required for sister chromatid cohesion, replication checkpoint activation and telomere length regulation [46-48]. Furthermore, it was shown that Ctf18-RLC can load and unload PCNA, however, this is most likely not its main function *in vivo* [49, 50]. ATAD5/Elg1-RLC ensures genome stability and recent evidence suggests that the human ATAD5-RLC, as well as the yeast Elg1-RLC, promotes PCNA unloading from chromatin.

While the canonical RFC is absolutely required for replication, none of the alternative RLCs are essential alone or in combination. Even the triple knock-out in yeast is viable, although growth rates are extremely impaired and cells are hypersensitive to DNA damaging agents. The role of ATAD5/Elg1 will be discussed in detail in the following chapter. For RAD17 and CTF18, I would like to refer the reader to excellent reviews by Majka *et al.* and Parilla-Castellar *et al.* [40, 45].



Process involved	DNA replication	DNA damage checkpoint	Cohesion establishment	Genomic stability (Replication & Repair)
***In vivo* molecular effect on clamp**	PCNA loading during replication	9-1-1 loading at DNA damage sites	unknown	Removal of PCNA during replication and repair
Phenotype of deletion mutant	•Lethal	•DNA damage checkpoint defect •Sensitivity to MMS, HU & UV	•Cohesion defect •DNA replication checkpoint defect •Abnormal telomere intranuclear positioning •Shortened telomere •Sensitivity to MMS & HU	•Gross chromosomal rearrangement •Elongated telomere •Cohesion defect •Sensitivity to MMS

Fig.4: Known functions of replication factor C (RFC) and replication factor C-like (aRFC) complexes. Summary of activities of *in vitro* loading and unloading data, which were derived from Bylund and Burgers 2005, Majka and Burgers 2003 (modified from Kubota *et al.* 2013 [51]).

ATAD5-RLC & ELG1-RLC

Elg1 was first identified in yeast genomic screens as an important factor for the maintenance of genome integrity (hence, it was named **e**nhanced **l**evels of **g**enome instability). Elg1 knock-out strains display, among other phenotypes, DNA damage sensitivity, gross chromosomal rearrangements, chromosome maintenance defects, replication defects, spontaneous DNA damage, enhanced homologous recombination events and elongated

telomeres [52-55]. Yeast *Δelg1* mutants have been well characterized, displaying synthetic growth defects with genes involved in sister chromatid cohesion (such as the cohesin subunits and Ctf4 that links replication and cohesion), checkpoint response (such as *mec1* (ATR), *rad53* (CHK2), *chk1* (CHK1), *sgs1* (WRN), *rad24* (RAD17), *dpb11-1* (TopBP1) and *dun1* (CHEK2)), replication fork restart and homologous recombination (such as mutants of the *rad52* group and the nucleases *mms4* as well as *mus81*). Elg1 seems to participate in sister chromatid cohesion together with the anti-establishment complex Wpl1/Pds5 [56] and a model was proposed in which Elg1-RLC and Ctf18-RLC promote momentary unloading and re-loading of PCNA when replication forks encounter cohesin complexes on the DNA [57, 58]. However, this model was recently challenged by Kubota *et al.*, claiming a general genome-wide PCNA unloading effect upon Okazaki fragment ligation on the lagging strand, rather than specific unloading at cohesion sites [59]. Deletion of Elg1 was also shown to increase telomere length, a phenotype that is dependent on telomerase activity and PCNA modification at K164 and K127. Furthermore, Elg1 mutants display hyper-silencing at telomeres [55], a phenomenon termed 'telomere position effect' (TPE).

Similarly to yeast Elg1, loss of mammalian ATAD5 causes genome instability, spontaneous DNA damage and increased levels of recombination. Homozygous deletion of the mouse homolog, FRAG1, leads to embryonic lethality but heterozygous mice display a haploinsufficiency phenotype with high levels of aneuploidy, apoptosis and predisposition to cancer [60, 61]. In human cells somatic mutations of ATAD5 were observed in 4.6% of sporadic endometrial tumors and micro-deletions including ATAD5 (located on chromosome 17q11.2) were also associated with increased cancer incidence, suggesting that ATAD5 is a tumor suppressor [60, 62].

Recently, both the yeast Elg1-RLC and the human ATAD5-RLC were proposed to function as major PCNA unloaders during normal replication [49, 50]. It was shown that unmodified as well as SUMOylated and ubiquitylated PCNA accumulates on DNA in *elg1* deletion mutants during S-phase. *'Ex-vivo'* experiments with immunopurified Elg1-RLC proved that the complex was able to promote PCNA unloading from chromatin, which was isolated from an *elg1* mutant strain [49]. Similarly, depletion of ATAD5 in human cells increases the amount of PCNA and ubiquitylated PCNA on chromatin and also extends the intensity and lifespan of PCNA foci in replication factories [50]. Interestingly, ATAD5 interacts with the UAF1-USP1 complex, which is responsible for the deubiquitylation of PCNA and FANCD2, a crucial factor for interstrand crosslink (ICL) repair [63]. ATAD5 may thus promote not only the unloading of ubiquitylated PCNA from chromatin but also its deubiquitylation mediated by the UAF1-USP1 complex. On the other hand, ubiquitylation of FANCD2 was not affected by ATAD5 knock-down, suggesting that ATAD5 is not directly involved in ICL repair. Due to its interaction with PCNA that is mediated by a conserved PCNA-interaction-peptide (PIP), it is not surprising that ATAD5 travels with the replication fork [64]. However, it seems that ATAD5 is not directly involved in replication, since no reduction in global replication fork speed or checkpoint activation was observed, merely a slight delay in S-phase progression [49, 50]. While ATAD5/Elg1 does not contribute directly to Okazaki fragment maturation, novel evidence suggests that yeast Elg1-RLC is involved in genome-wide replication-coupled unloading of PCNA after Okazaki fragments have been successfully ligated by the Cdc9 ligase [59]. Nevertheless, Elg1/ATAD5-RLC does not seem to be the only factor able to unload

PCNA, as indicated by ChIP-sequencing experiments and only a weak growth retardation phenotype in Elg1/ATAD5 depleted cells [59].

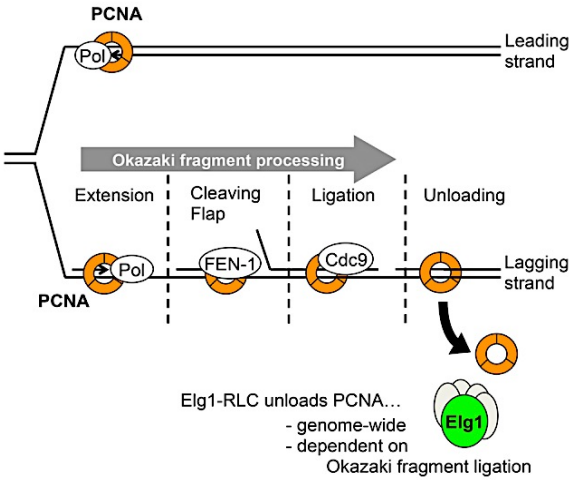


Fig.5: The Elg1-RFC2-5 complex unloads PCNA following Okazaki fragment ligation. Schematic depiction of PCNA trimer during replication of the leading and the lagging strand. The Elg1-RLC is responsible for PCNA unloading after successful ligation of Okazaki fragment by the Cdc9 ligase on a genome-wide level (modified from Kubota *et al.* 2015 [59]).

Despite these recent advances in the field, *in vitro* assays, which would provide genuine evidence that the Elg1-RLC/ATAD5-RLC complexes have enzymatic activity in unloading PCNA from chromatin, are still lacking. The reason for this may be missing essential yet unknown co-factors or posttranslational modifications, or possibly redundancy with other clamp unloaders, such as CTF18-RLC. Furthermore, it still remains enigmatic how unloading of PCNA or modified forms of PCNA contributes to genomic stability, since it does not seem to disturb replication and Okazaki fragment maturation *per se*. Novel results indicate that genome-wide PCNA unloading is required to promote genome stability, rather than unloading at specific DNA structures and locations in the genome, such as DNA repair or cohesion sites [65]. This may suggest that ATAD5 is needed post-replicatively to avoid recruitment of interaction partners that initiate inappropriate DNA repair or recombination events [58].

Interestingly, Elg1 was shown to interact preferentially with SUMOylated PCNA via its three N-terminal SUMO-interacting-motifs (SIM) and $PCNA^{SUMO}$ accumulates on the chromatin in the absence of Elg1 [66]. SUMO-modified PCNA recruits the DNA helicase Srs2 to replication forks, where it prevents unscheduled recombination events, as described earlier [34]. Further, physical interactions were identified between Elg1 and the SUMO E2 Ubc9 and with the SUMO-Targeted Ubiquitin Ligase (STUbL) complex Slx5/Slx8, that selectively ubiquitylates (poly)SUMOylated proteins to mark them for degradation [67-69]. While SUMO has been proposed to act as a general 'glue', holding together different components of the same pathway to coordinate enzyme activity [38], STUbL may then be recruited to these sites and specifically degrade SUMOylated factors, once repair is completed.

Replication stress

The replication fork is a fragile structure, especially due to the generation of ssDNA during the process of helix unwinding. ssDNA is vulnerable to chemical and nucleophilic attack,

formation of secondary structures (such as hairpins, triplexes, G-quadruplexes, etc.) and binding of inappropriate factors. Physical replication blocks in the form of bulky DNA lesions or interstrand crosslinks, secondary DNA structures or protein-DNA adducts, as well as deprivation of essential replication factors (such as nucleotides, replication machinery components, histones and histone chaperones) result in fork stalling [70]. While MCM helicase continues to unwind the DNA it uncouples from the stalled replication fork, leading to the generation of long stretches of ssDNA [71, 72]. Eventually, forks may collapse, resulting in the formation of one-ended double strand breaks (DSBs) [73, 74]. While replication barriers may be confined to only one strand (leading or lagging), resulting in strand uncoupling, imbalance or deprivation of replication factors will cause DNA synthesis to cease at both strands.

SSBs and longer stretches of ssDNA are natural DNA repair intermediates and also transiently generated by topoisomerases. Encounters of an active replication fork with a SSB has been proposed to result in a 'run off', where the replication machinery disassociates or runs off the DNA, consequently leading to the formation of a passive DSB [75]. Another very common source of DNA damage is created by misincorporation of ribonucleotides (rNTPs) into the DNA that was shown to stall replicative polymerases [76]. Topoisomerase I (TopoI)-mediated processing of rNTPs causes accumulation of non-ligatable nicks that in turn further increase replication stress [77, 78]. Additionally, collision of the replication machinery with ongoing transcription, for instance at 'early replicating fragile sites' or 'common fragile sites', can cause replication fork collapse [79]. Topological stress, resulting from tethering of transcribed genes to the nuclear pore, can cause replication fork collapse even before replication and transcription machineries collide [80, 81]. Other factors that induce replication stress include DNA accessibility and activation of oncogenes, such as HRAS, MYC and cyclin E, possibly by increased origin firing and deprivation of the nucleotide pool [82-85].

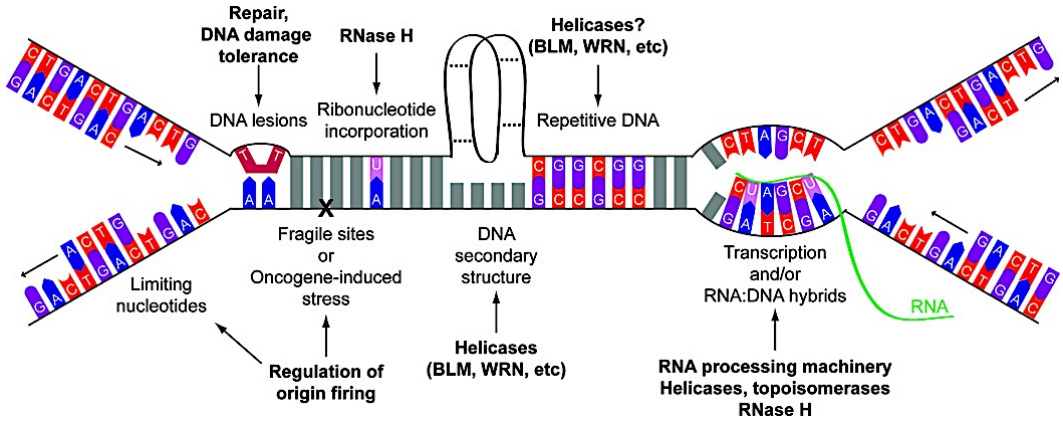


Fig.6: Replication stress and its causes. A variety of conditions or obstacles can slow down or stall DNA replication forks. These include nucleotide shortage, certain DNA lesions, mis-incorporated ribonucleotides, repetitive DNA elements, transcription complexes as well as RNA–DNA hybrids, secondary DNA structures, early-replication or common fragile sites, and oncogene-induced replication stress. Key resolution pathways are indicated in bold (from Zeman and Cimprich, 2013 [70]).

GENOME INTEGRITY

As mentioned earlier it is crucial to maintain the integrity of the genetic information, not only during replication, but throughout the entire lifetime of a cell. However, our DNA is constantly exposed to different types of exogenous and endogenous damaging agents, inducing tens of thousands of DNA lesions every day [86]. If left unrepaired, these lesions can hamper or deregulate replication and transcription, cause gene mutations or loss of genetic information, which can ultimately lead to malignant transformation of a cell. In order to protect the genome from the deleterious effects of the various types of DNA damaging agents, a network of repair pathways has evolved [87].

DNA damage

The DNA bases are highly vulnerable to chemical modifications, which can be converted into permanent mutations by means of faulty repair or replication errors. Very common types of DNA damage are induced by environmental sources, such as ultraviolet (UV) light, ionizing radiation (IR), chemicals or tobacco smoke [87]. The three major forms of endogenous DNA damage are caused by spontaneous depurinations, deamination and cellular reactive metabolites. Spontaneous hydrolysis of the N-glycosidic bond results in the formation of an abasic site, while single-strand breaks (SSBs) and oxidations or alkylations of bases are induced by reactive oxygen and nitrogen species (ROS and RNS, respectively) or other toxic metabolic intermediates [88].

Depending on the type of lesion its abundance and location, DNA injury can either be primarily mutagenic, cytotoxic or cytostatic. The consequences can also be diverse, causing cancer or other disease phenotypes, cell death or senescence. The genomic maintenance apparatus, consisting of DNA repair, damage tolerance and cell cycle checkpoint pathways, controls DNA damage and determines the fate of a cell [89, 90].

DNA damage response

Upon detection of DNA damage, cells evoke several pathways to stall the cell cycle to allow sufficient time for repair of the lesions. A permanent arrest of the cell cycle antagonizes the onset of cancer, since it prevents the accumulation of mutations and the subsequent transformation into malignant cells [87], but it is also associated with ageing.

DNA damage response (DDR) is a complex network of pathways activated in response to DNA insults. In addition to signaling to recruit the DNA repair machinery, DDR may induce temporary cell cycle arrest, senescence or even cell death via apoptosis, depending on the damage status. Swift and precise signaling is required to organize a fine-tuned and appropriate response to the specific type of DNA damage.

ATM and ATR

The ataxia-telangiectasia mutated (ATM) and ATM-and-Rad3-related (ATR) kinases belong to the phosphatidyl-inositol 3-kinase related kinase (PIKK) family and play a major role in the

DDR as primary signal transducers [90]. Both serine/threonine kinases phosphorylate a great number of substrates in response to genotoxic stress and, although they share some substrates and cooperate to induce the DDR, they show distinct activation patterns.

ATM regulates the immediate response to double strand breaks in the DNA. Activation of ATM enables phosphorylation of downstream effector and signaling proteins, including the histone variant H2AX and the checkpoint kinase 2 (CHK2). Phosphorylated H2AX (γH2AX) serves as a molecular adaptor for proteins required in the repair and signaling processes, and binds 53BP1 as well as BRCA1, both factors mediating different DSB repair branches [91]. Another substrate of ATM is p53, which is involved in the regulation of DNA repair and cell cycle control together with CHK2 [92]. In addition to induction by DNA damage, ATM can also be activated directly in response to H_2O_2 treatment by dimer formation via Cys2991 oxidation. This eventually leads to activation of tumor suppressors and induces autophagy, suggesting that ATM is crucial for a cellular oxidative stress defense program [93].

ATR is one of the key replication-stress-response kinases, and once activated it phosphorylates substrates that help cells to survive and complete replication under stress conditions. It is recruited to single stranded DNA (ssDNA), which is generated as a result of replication stress when polymerases are stalled and helicases continue to unwind DNA, but also upon resection of DNA from the site of a DSB [94]. Stretches of ssDNA are coated and protected by replication protein A (RPA), which is recognized by ATR via the ATR interacting protein (ATRIP) [95]. In response, ATR becomes activated and phosphorylates RPA (Ser33) and CHK1 (Ser345) [96, 97] to mediate cell cycle stalling and suppression of late origin firing.

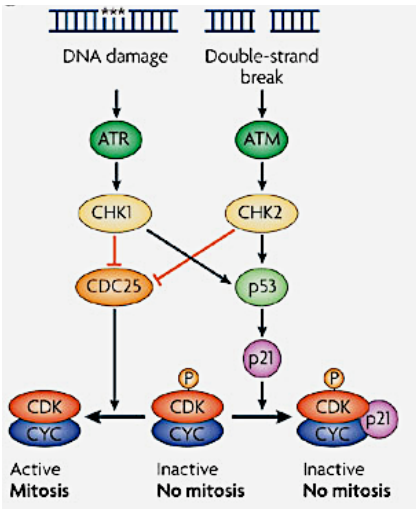


Fig.7: DNA damage response mediated by ATM and ATR. ATM and ATR are DNA damage-sensing kinases that activate the CHK1 and CHK2 protein kinases. CHK2 in turn targets the CDC25 phosphatase for its destruction or inhibition of its nuclear import. This prevents activation of the CDK/cyclin complex and therefore cell cycle progression and/or mitotic entry. Additionally, the CDKi $p21^{Kip1}$ is activated by p53 and inhibits CDK activity (modified from De Veylder, Beeckman & Inzé, 2007 [98]).

CHK1 and CHK2

Important substrates of ATM and ATR are the checkpoint kinases CHK2 and CHK1, which play major roles in downstream signaling after DNA damage and replicative stress [99]. The main function of these signal transducers is to propagate the DNA damage response and translate it into cell cycle regulation, DNA repair initiation or apoptosis. ATR-dependent activation of CHK1 allows phosphorylation of downstream targets, such as CDC25 phosphatases [100] and is thus essential for cell cycle arrest in S and G2/M phases. Additionally, CHK1

regulates replication dynamics, primarily by inhibiting origin firing [101]. CHK2 is preferentially phosphorylated by ATM in response to DSBs and triggers G1/S arrest and DNA repair by activating CDC25 family members, p53 and BRCA1 [102, 103].

Inhibitors of ATR and CHK1 are tested as possible therapeutics in cancer therapy due to a synthetic lethal interaction with oncogene-induced replication stress or loss of p53 [104, 105].

Cell cycle arrest

Upon detection of DNA damage through the above-mentioned DDR, an arrest of the cell cycle is triggered at three checkpoints (G1/S, intra-S and G2/M). This mediates a delayed transition into the next cell cycle phase and allows repair of the damaged sites.

In G1, the active cyclinD/CDK4,6 complex phosphorylates the retinoblastoma (RB) protein, which releases E2F [13, 106, 107], a transcription factor for cyclinE and CDC25A [102, 108], to promote transition into S-Phase. In response to high amounts of DNA damage, the tumor suppressor p53 becomes activated by ATM-mediated phosphorylation at Serine 15. Downstream targets of p53 are the CDK inhibitors $p16^{INK4a}$ and $p21^{Cip1}$, which induce a permanent arrest of the cell cycle. Temporary arrest of the cell cycle before entering S-phase is mediated through transient phosphorylation of CDC25A by CHK2/CHK1 [109-111].

The intra-S checkpoint is activated by both DNA damage and replication stress, mainly through the ATR-CHK1 pathway [112]. CHK1-mediated phosphorylation of CDC25A marks the checkpoint phosphatase for degradation. This leads to abolished activation of CDK1 and CDK2, resulting in reduced origin firing and delayed progression into S-phase [113, 114].

The G2/M checkpoint prevents mitotic entry in response to DNA damage, that remained undetected upon completion of S-phase, or that occured during the G2 phase of the cell cycle [115]. Dephosphorylation of cyclinB/CDK1 by CDC25 phosphatases usually activates the complex after faithful replication [116]. In response to DNA damage, activation of cyclinB/CDK1 is prevented mainly by CHK1-mediated phosphorylation of CDC25 [117]. In parallel, stabilization of p53 promotes transcription of CDK inhibitors and prevents the progression into mitosis.

If the load of DNA damage is too high to cope with normal cellular functions, cells either die or induce a permanent arrest of the cell cycle, called senescence. Senescent cells are unable to replicate, however, they are still metabolically active and adopt an immunogenic phenotype. Apoptosis is a form of programmed cell death induced by the cell itself (intrinsic) or by extrinsic signals from other cells. It is a highly regulated and controlled process that differs from necrosis, which is a form of traumatic cell death resulting from massive cellular injury.

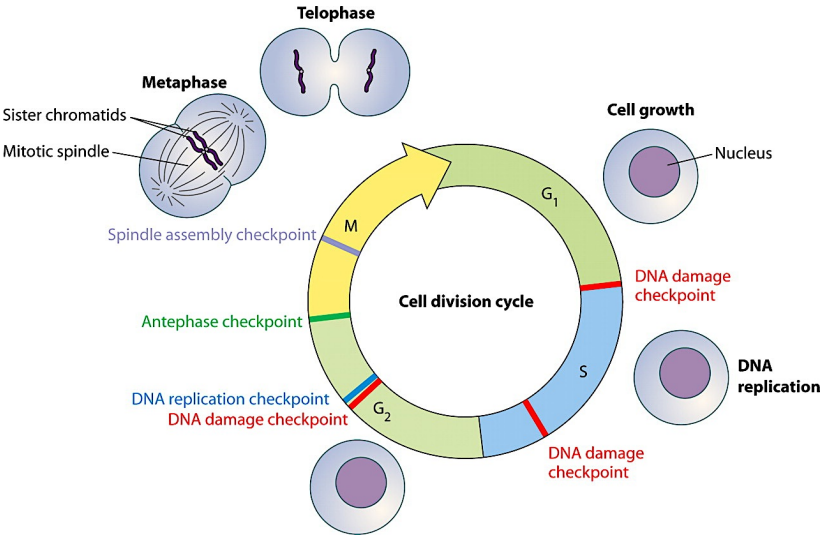


Fig.8: Cell cycle checkpoints. The DNA damage, replication and spindle assembly checkpoints ensure integrity of the DNA during different phases of the cell cycle (from Chin and Yeong, 2010 [118]).

DNA repair

Arrest of the cell cycle provides the cell with sufficient time to complete DNA repair and thus helps ensure the transmission of intact genetic information to the daughters. Several specialized DNA repair pathways take care of the various types of DNA lesions before they can be converted into mutations during replication. Base excision repair (BER) and single-strand break repair (SSBR) are the most frequently used pathways in human cells to repair small alterations of DNA bases, such as oxidations and alkylations. Nucleotide excision repair (NER), on the other hand, removes gross distortions of the DNA double helix, such as bulky adducts and UV-induced base-dimers, by excising stretches of about 30 nucleotides surrounding the lesion. Homologous recombination (HR) and non-homologous end joining (NHEJ) act in concert to take care of extremely cytotoxic DNA double-strand breaks (DSBs) caused for instance by ionizing radiation, free radicals and chemicals, or as a consequence of replication stress. Mismatch repair (MMR) scans the newly synthesized DNA for inaccuracies and removes mismatched bases introduced by the replication machinery. DNA-protein crosslinks are replication- and transcription-blocking lesions, which are repaired by dedicated proteases, such as yeast Wss1 and mammalian Spartan (SPRTN, DVC1). Alternatively, depending on the type and size of the protein cross-linked to DNA, tyrosyl-DNA phosphodiesterases (TDP1/2) or the NER and HR pathways can eliminate these cytotoxic lesions. DNA repair mechanisms are discussed in detail in several comprehensive reviews [87, 89, 119-121].

Failure to maintain integrity of the genomic material due to errors or insufficiency of the DNA repair process may lead to accumulation of mutations, which fuel tumor progression. Furthermore, mutations in proteins involved in the above mentioned pathways may cause faulty DNA repair and have been linked to genomic instability syndromes with predisposition to various types of cancer and several other human diseases [122]. Genomic instability has thus been included as a hallmark of cancer.

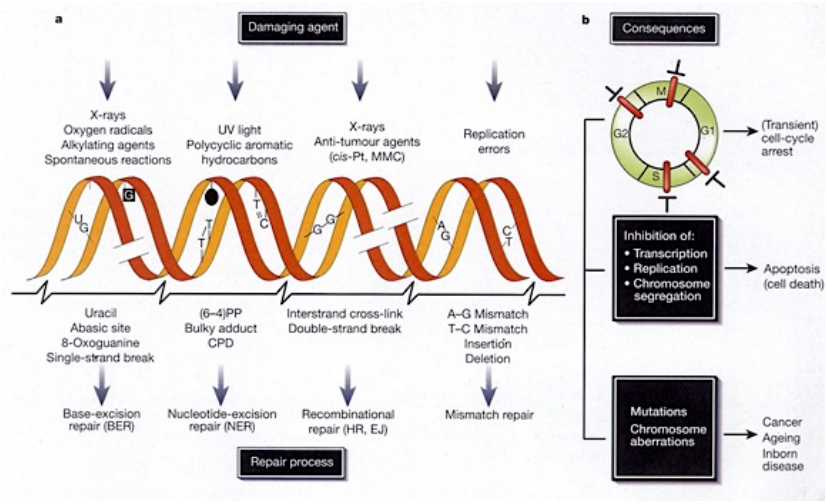


Fig.9: DNA damage, repair mechanisms, and consequences. **a)** DNA damaging agents induce specific lesions that are repaired by dedicated repair mechanisms. **b)** DNA damage causes cell cycle arrest, inhibition of DNA replication or permanent mutations. Abbreviations: cis-Pt (cisplatin), MMC (mitomycin C); (6-4) PP (6-4 photoproduct), CPD (cyclobutane pyrimidine dimer) (from Hoeijmakers *et al.*, 2001 [89]).

Base Excision Repair

Small DNA modifications that frequently occur through oxidation, alkylation or deamination of the bases are repaired by base excision repair (BER). These lesions often cause only minor distortions of the DNA double helix structure, but they can be mutagenic or less stable, risking spontaneous base loss. BER acts across the entire genome and thus has to be a highly sensitive and coordinated process. The basic steps of BER include 1) recognition and excision of the modified base by dedicated DNA glycosylases, 2) incision of the abasic (AP) site, 3) end processing by an endonuclease or lyase and 4) polymerase-mediated gap filling followed by ligation.

Damage-specific DNA glycosylases scan the DNA for modified bases as they move along the DNA double helix. To date, eleven human glycosylases have been identified, which display a wide range of substrate specificities and are subdivided into four distinct superfamilies; helix-hairpin-helix (HhH) glycosylases, uracil DNA glycosylases (UDGs), 3-methyl-purine glycosylases (MPG) and endonuclease VIII-like (NEIL) glycosylases [123]. Many of these glycosylases have overlapping substrate specificities and are redundant, as indicated by the fact that mice with a single knockout of either of these glycosylases display mild phenotypes (with the exception of TDG), while double and triple knockout mice are often very sick [88, 124, 125].

An amino acid side chain of the DNA glycosylase (*e.g.* in case of OGG1: Phe 114 and MYH: Tyr 165) reaches into the minor groove of the DNA helix and is used to search for vulnerable sites, disrupting pairing of non-Watson-Crick bases pairs. The modified base is flipped out and extruded into the exo-site of the enzyme. After verification of the base aberration it is passed on to the active site pocket where the N-glycosidic bond between the base and the DNA backbone is cleaved, leaving behind an apurinic/apyrimidinic (AP) site [126].

Monofunctional glycosylases, such as MYH, UNG, SMUG1, MBD4, TDG and MPG, remove the base by nucleophilic attack on the C1' of the sugar, using a deprotonated water molecule to cleave the N-glycosidic bond. Further processing by the apurinic/apyrimidinic endonuclease 1 (APE1) is required for cleavage of the DNA sugar-phosphate backbone at the AP site to create suitable DNA ends for subsequent repair steps, since the polymerase requires a 3’ hydroxyl (3'OH) group to be able to replace the excised nucleotide in a 5’ to 3’ direction. Additionally, a 5’ phosphate group (5'P) is needed to reconnect the DNA backbone and seal the break. These

termini are generated by end-processing enzymes such as polynucleotide kinase 3'-phosphatase (PNKP) [123]. Bifunctional glycosylases, such as OGG1, NTH1, NEIL1, NEIL2 and NEIL3, possess an intrinsic AP-lyase activity that cleaves the sugar-phosphate backbone via β– or βδ–elimination reactions. However, APE1 and PNKP are still required to further process the ends in order to create suitable ends for the subsequent gap filling and ligation reactions [127].

The majority of the glycosylases actually bind the newly generated AP-site with higher affinity than the damaged base. This way they protect the noncoding and chemically unstable AP-site until further processing is enabled by cleavage of the phosphodiester bond [128-130].

Replacement of the excised nucleotide(s) can be achieved by short-patch (SP) or long-patch (LP) repair. Mammalian cells predominantly use the SP pathway [88], which involves the replacement of a single nucleotide, primarily by DNA polymerase β (Polβ), and subsequent ligation by ligase IIIα (LigIIIα). The X-ray repair cross-complementing 1 (XRCC1) protein is crucial for BER, binding to the single-strand break and acting as a scaffold protein to coordinate the downstream repair processes [131-133]. Long-patch BER includes re-synthesis of 2 to 12 nucleotides at the damage site and relies on Polβ for the incorporation of the first nucleotide [134]. Then replicative polymerases Polδ or Polε together with PCNA and its loading factor (RFC) take over to complete the polymerization. The flap endonuclease 1 (FEN1) excises the displaced oligonucleotide and DNA ligase 1 (LigI) subsequently seals the nick. Due to the requirement for these factors, this pathway is predominantly active in proliferating cells [135, 136].

Oxidized bases are DNA lesions, which are mainly processed by the DNA glycosylase OGG1 and endonuclease III (hNTH1). Both are bifunctional glycosylases, which carry an additional intrinsic 3' AP lyase activity that enables them to cleave the DNA phosphodiester bond at the AP site independently of APE1. However, APE1 is still required for processing of the blocked 3' end to allow DNA synthesis over the gap by Polβ [41]. MYH is a unusual glycosylase, that recognizes and excises an unmodified adenine (A), which has been mispaired with 8-oxoguanine due to misincorporation by replicative polymerases. It is thus also involved indirectly in the repair of oxidized bases. I would like to refer to the chapter 'Damage prevention and base excision repair of 8-oxoguanine' of this thesis for a detailed description of OGG1- and MYH-mediated BER reactions and chapter 'Oxidative stress and disease' for further information on oxidative DNA damage.

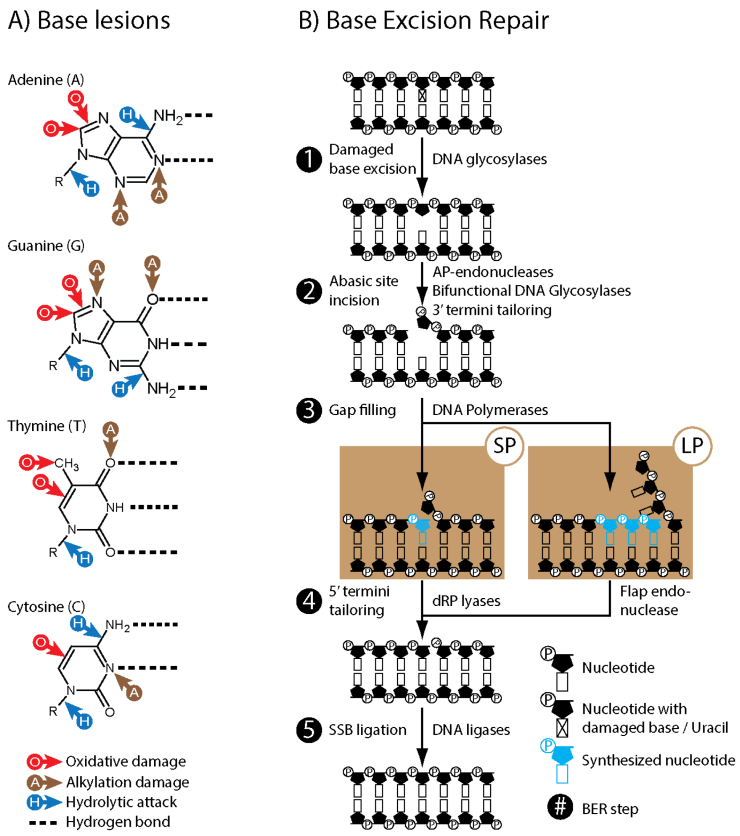


Fig.10: Base lesions and base-excision repair. A) Base lesions: Common sites of oxidation (red arrow), alkylation (brown arrow) and spontaneous hydrolysis (blue arrow) on the different bases of the DNA: adenine, guanine, thymine and cytosine. **B**) Base-Excision Repair: 1 to 5 major repair steps and involved enzymes as well as the enzymatic activities are indicated. (from Hanssen-Bauer *et al.*, 2012 [137]).

Single-Strand Break Repair

Single-strand break repair (SSBR) shares many properties with the BER pathway, differing only in the early damage recognition steps. While BER is initiated by recognition of a damaged base by dedicated DNA glycosylases, SSBs are bound with high affinity by an enzyme named poly(ADP-Ribose)polymerase 1 (PARP1). Upon binding to DNA breaks, PARP1 activity is rapidly stimulated more than 500-fold and catalyzes the synthesis of long chains of poly(ADP-Ribose) (PAR) units on itself and other acceptor proteins. PARylation serves as a platform to recruit downstream repair factors, such as the scaffold protein XRCC1, and accelerates repair of SSBs, predominantly at early times following SSB induction. SSBs can arise either directly, *e.g.* as a consequence of oxidative DNA damage resulting in the disintegration of desoxyribose, or indirectly as transient intermediates of BER or other DNA repair actions. Especially in the context of 'direct' SSBs that arise in the complex environment of chromatin, PARP1 activity might be required as a dedicated 'sensor' system to accelerate lesion detection and promote their efficient repair. However, a requirement of PARP1 can also be anticipated for the repair of scheduled SSBs that become 'uncoupled' from BER and possibly other repair pathways. A more detailed discussion of the properties of PARP1 and other family members can be found in chapter 'ADP-Ribose Metabolism' of this thesis.

After recognition of the SSB, end-processing by APE1 and/or RNKP is required to generate suitable ends (3'-OH and 5'P) for gap filling and subsequent ligation. DNA breaks that arise as a consequence of abortive Topoisomerase 1 (Top1) activity consist of 3'- and 5'- termini covalently attached to Top1 and AMP, respectively. These lesions require additional processing

by the tyrosyl-DNA phosphodiesterase 1 (TDP1) as well as Aprataxin (APTX). RNaseH2-mediated excision of ribonucleotides that have been misincorporated into the DNA results in the formation of 5'-ribonucleotide termini that might also require APTX activity. Gap filling by Polβ or Polδ/ε and subsequent ligation by LigIIIα or LigI is carried out in a similar fashion as during BER, and depends on short- or long-patch subpathway choice (reviewed in [138, 139]).

Double-Strand Break Repair

DNA double-strand breaks (DSBs) are highly cytotoxic lesions, because they can cause deleterious genomic rearrangements. Environmental factors, such as ionizing radiation (IR), but also endogenous factors, like reactive oxygen species (ROS) or cleavage by cellular endonucleases, can lead to the formation of DSBs. These so-called two-ended DSBs may trigger a different damage response as compared to the one-ended DSBs generated by replication fork collapse. Homologous recombination (HR) and non-homologous end-joining (NHEJ) are the two major pathways that act to repair DSBs, while single-strand annealing (SSA) is a minor repair pathway. HR is restricted to the S- and G2-phase of the cell cycle, since this pathway requires the presence of a sister chromatid for error-free repair. NHEJ on the other hand is active throughout the cell cycle, but it is rather error prone [89, 140, 141].

Phosphorylation of the histone H2AX (γH2AX) spreading rapidly away from the two-ended DSB in both directions enables the recruitment of DSB response factors [142, 143]. MRN and ATM act in concert to phosphorylate H2AX upon sensing of two-ended DSBs, while phosphorylation in response to replication stress is dependent on ATR [144].

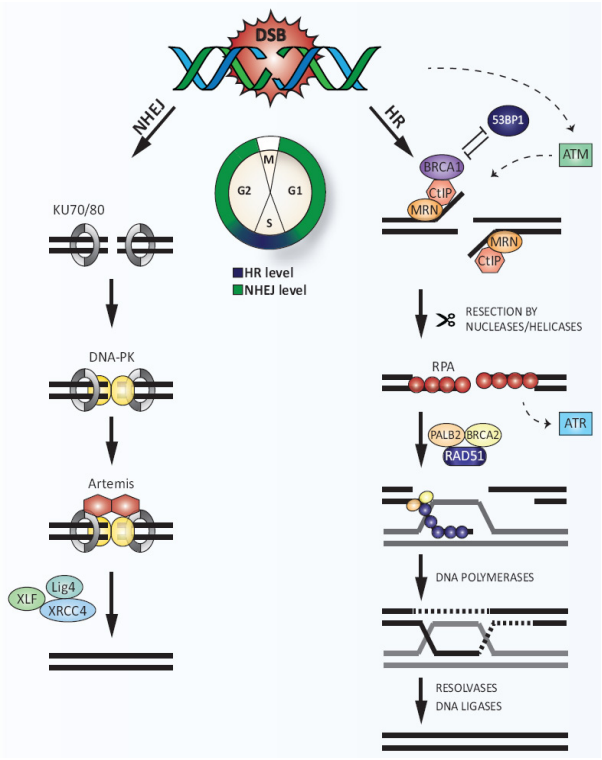


Fig.11: DNA double-strand break (DSB) repair mechanisms. DSBs are predominantly repaired by NHEJ or HR. NHEJ is active throughout the cell cycle, but mainly during the G1 and G2 phases, whereas HR peaks in S phase when a sister chromatid is available. Rapid binding of the Ku70/80 heterodimer to DNA ends promotes NHEJ by recruiting DNA-PKcs. DNA ends are processed by Artemis, followed by religation mediated by XLF, Ligase IV (Lig4) and XRCC4. Alternatively, the MRN complex, which competes with Ku70/80 for binding of the DSB, initiates resection together with CtIP to promote HR. The NHEJ factor 53BP1 antagonizes BRCA1 in DSB resection. Further resection and subsequent formation of RPA-coated ssDNA stimulates ATR activation. BRCA2 together with PALB2 mediates the displacement of RPA by RAD51, allowing strand invasion into the homologous DNA template and second end capture. This leads to the formation of a double Holliday junction, which is processed by resolvases. Finally, the DNA is sealed by ligases (from Hühn *et al.*, 2013 [145]).

Homologous Recombination

HR is an accurate DSB repair pathway that uses the homologous information on a sister chromatid as a template for repair and takes place in three core steps: 1) end resection, 2) strand invasion and 3) resolution. The MRE11/RAD50/NBS1 (MRN) complex initiates DSB repair by immediately binding the broken ends and inducing endonucleolytic cleavage of the DNA at some distance from the DSB [146, 147].

The initial end resection, called 'end clipping', is promoted by the structure-specific nucleases MRE11 and CtIP, leading to the degradation of about 20 base pairs in mammalian cells. In a second step 'extensive resection' is achieved by a set of different helicases and nucleases, such as DNA2, BLM, WRN, CtIP and EXO1. This ultimately leads to the generation of single-stranded 3' overhangs that can be several hundred base-pairs long. The ssDNA is rapidly coated by RPA and subsequently BRCA2 helps to replace RPA with the RecA type recombinase RAD51 [148, 149]. RAD51 is a DNA-dependent ATPase that catalyzes strand invasion in search of a homologous sequence on the undamaged sister chromatid serving as a template for the DNA polymerase [150]. Two DNA binding domains within RAD51 mediate tethering of the resected 3' ssDNA overhang to the donor sequence with minimal homology of approximately 8 bp, which likely become available through intrinsic 'DNA breathing' especially at thermally more unstable AT-rich regions. *In vitro*, cooperative sliding of the presynaptic nucleofilament within a range of 60-300 bp on the dsDNA and 'intersegmental contact sampling' of multiple DNA regions at the same time, are believed to mediate homology identification (reviewed in [151]). ChIP experiments of RAD51 at various time points after DSB induction have revealed that homology probing favors intrachromosomal recombination and that homology search is mostly driven by spatial proximity in a three-dimensional setting [152]. In S, G2 and M close proximity is given by the architecture of the replication fork, sister chromatid cohesion and alignment of homologous chromosomes, respectively. Transient chromatin opening, *e.g.* by nucleosome shifting or eviction, could accelerate homology probing at distal regions [153]. This led to the postulation of an 'accelerated random search model', stating that homology search is a random probing mechanism that is accelerated by simultaneous multiple contacts with different DNA segments, sliding of the RAD51-DNA filament on the DNA and induced chromatin mobility [151].

Upon invasion, a so-called D-loop structure is formed, which is composed of the ssDNA filament and the double stranded sister chromatid. The D-loop is converted into a (double) Holiday junction (HJ) when both strands have paired with the homologous sequences [154]. Different endonucleases resolve HJs, forming either crossover or non-crossover products, depending on the endonucleolytic cleavage pattern [155, 156].

BRCA1 and BRCA2 are crucial for HR and frequently lost in breast cancer. While BRCA1 is required for DNA end-resection and the regulation of pathway choice, as well as recruitment of downstream DNA repair factors, BRCA2 it mediates RAD51 nucleofilament formation and strand exchange. The BRC domains of BRCA2 are required to disrupt self-assembled RAD51 oligomers and promote loading of RAD51 in monomeric form onto ssDNA. The C-terminal domain of BRCA2 promotes RAD51 nucleofilament assembly and participates in strand invasion [157-159].

BRCA1/2 mutant tumors display an obvious mutational spectrum (signature 3) that is associated with elevated levels of large (longer than 3 bp) insertions and deletions with overlapping microhomology at breakpoints. Alternative non-homologous end-joining (alt-NHEJ)

and single-strand annealing (SSA) may serve as backup pathways when HR is impaired and could explain this mutation signature [160, 161].

Another protein involved in HR is RAD54, an Snf2/Swi2 helicase family member that stabilizes RAD51 filaments with homologous DNA and increases the mobility of DSBs, possibly through nucleosome remodeling. Importantly, RAD54 is also required for the coordinated removal of RAD51 from ssDNA while extending the D-loop to generate a template for HR-associated DNA synthesis [162]. Chromatin modifiers, such as INO80 and SWI/SNF, as well as DDR mediators, like ATR, CHK2 and 53BP1, are likely important for increased chromatin dynamics upon DSB initiation [163, 164]. Interestingly, ChIP signals of yeast Rad51 and γH2A overlap, suggesting that γH2AX does not spread in a linear fashion from the DSB as previously assumed [165].

Single strand annealing

SSA is a subpathway of HR that utilizes the homologous sequences of the complementary strand for strand annealing, instead of a sister chromatid and therefore does not require a donor sequence. Upon recognition of the DSB by the MRN complex, extensive resection occurs until regions of homology are exposed on either side of the DSB. These homologous regions are then paired, the overhangs are removed and the ends ligated [120]. Since the SSA mechanism causes loss of genetic material, it is highly error-prone. In contrast to HR, SSA does not require strand invasion; the actions of BRCA2 and RAD51 are therefore dispensable for this repair pathway. Consequently, loss of the tumor suppressor gene BRCA2 stimulates the use of SSA [166]. In contrast, RAD52 and Rad1-Rad10 (ERCC4-ERCC10) are involved in SSA in mammalian cells [167] and thus loss of RAD52 induced synthetic lethality in HR-deficient cells (BRCA1, BRCA2, PALB2) [161].

Non-Homologous End Joining

At times, when no sister chromatid is available, as during G0- and G1-phases of the cell cycle, NHEJ is the predominant repair pathway for two-ended DSBs. It is tightly regulated due to its error-prone nature, however, its activity is also necessary for biological processes, *e.g.* during V(D)J recombination and class switch recombination (CSR) as part of the immune response. Classical NHEJ (c-NHEJ) is initiated by binding of the broken ends by the KU70/80 heterodimer, which acts as a scaffold, recruiting DNA-PK catalytic subunit (DNA-PKcs) to build the DNA-PK complex [168, 169]. Once activated, DNA-PK phosphorylates itself, as well as other NHEJ proteins, and holds the broken DNA ends in close proximity. Several factors, such as MRN, Artemis, PNK and WRN, are involved in processing of the broken ends in order to generate blunt ends that are ready for ligation [170]. Processing of the ends may lead to loss of nucleotides; NHEJ is therefore an error-prone pathway. Finally, rejoining of the DNA ends is accomplished by XRCC4 in conjunction with DNA ligase IV [171].

Alternative NHEJ (alt-NHEJ), also known as microhomology-mediated end-joining (MMEJ), is a less efficient pathway that takes place in the absence of KU70/80. It utilizes 3-16 base pair

homologies at the broken DNA ends in order to align them and enable re-ligation by DNA ligase III [172, 173].

Pathway choice

The choice whether a cell uses HR or NHEJ to repair a DSB is tightly regulated. Cell cycle dependent expression, degradation and posttranslational modification of repair factors is important to suppress HR outside of S- and G2 phases when no sister chromatid is available for faithful repair. Upon DSB detection, the histone H2AX gets phosphorylated, marking nucleosomes over megabase regions flanking the break (however, not in a linear fashion), serving as a platform for recruitment of DNA signaling and repair proteins [174].

A major determinant, dictating pathway choice and repair outcome, is DNA end-resection. Binding of the NHEJ factors KU70/80 at free DNA ends blocks access of the resection machinery and therefore inhibits HR. Similarly, 53BP1 prevents CtIP from accessing DNA ends and directs repair through canonical NHEJ (c-NHEJ) by tethering broken DNA ends in close proximity and facilitating their ligation in a process termed 'synapsis' [175]. This end-protecting function of 53BP1 depends on ATM-mediated phosphorylation and promotes recruitment of RIF1, PTIP and the nuclease Artemis [176-179]. 53BP1 and RIF1 further inhibit recruitment of BRCA1 specifically in G1 to avoid HR when no sister chromatid is present [180, 181]. Recently, REV7 (also known as MAD2L2) was shown to promote c-NHEJ downstream of RIF1 by inhibiting 5'-3' end-resection [182, 183]. Consequently, absence of these factors increases HR frequency or even restores HR in certain cases. This is important, especially for the selective treatment of HR-deficient cells with PARP inhibitors, because restored HR function might induce resistance to these agents [184, 185].

Conversely, NHEJ can be inhibited by HR, for instance through clipping KU70/80 from DNA ends by MRN and CtIP [141]. S-phase-dependent phosphorylation of MRN complex members BRCA1, CtIP, EXO1 and BLM by cyclin-dependent kinases (CDKs) and ATM promotes efficient end resection [186, 187]. CtIP is central in this process: protein levels are upregulated upon progression through S/G2 and CDK-dependent phosphorlytion promotes complex formation between CtIP, MRN and BRCA1 and activates resection [188, 189]. Other posttranslational modifications that regulate protein activity are acetylation, SUMOylation, neddylation and polyribosylation. RNF111/UBE2M-dependent neddylation of BRCA1 for instance inhibits end resection, while poly(ADP-)ribosylation of BRCA1 promotes resection and thus favors HR [190, 191].

Negative regulators of HR are helicases, such as Srs2, PARI, RECQL5, BLM, FANCJ and FBH1, which dismantle RAD51 nucleofilaments in a process that requires ATP hydrolysis [23, 35, 160, 192-196]. Additionally, D-loops can be displaced by a subset of helicases, such as RTEL1 and FANCM, thereby preventing crossover events and promoting synthesis-dependent strand-annealing (SDSA) [197-199]. The TLS polymerase Polυ also acts as an anti-recombinase, by limiting the formation of RAD51 nucleofilaments at a presynaptic step [160, 192].

Repair of one-ended DSBs

One-ended DSBs can form either by a polymerase 'run-off' at a SSB, as discussed earlier, or by collapse of ssDNA exposed at stalled replication forks. Moreover, active cleavage of fork structures by endonucleases such as MUS81 has been proposed to be required for fork collapse and leads to DSBs formation [200]. The benefit of such an action could be to generate free DNA ends for HR-mediated strand invasion and replication fork restart.

Break-induced replication (BIR) is the proposed mechanism for HR-mediated repair of one-ended DSBs [201] and starts with resection of the free DNA end, creating a 3' ssDNA overhang. RAD51-mediated strand invasion, D-loop formation and eventually Holiday junction (HJ) resolution allow faithful repair. HR-deficient cells are hypersensitive to replication stress, but also cells with a defect in NHEJ display sensitivity, implying NHEJ as a backup mechanism for replication-dependent DSB repair [75].

DNA damage bypass

Tolerance of DNA lesions can be favored under certain circumstances, *e.g.* when the repair process would interfere with replication and cause fork collapse. In order to protect the genome, the lesion is bypassed and repaired later on. Translesion synthesis (TLS) allows replication through damaged sites due to specialized DNA polymerases, which have more flexible base pairing properties than replicative polymerases. Their active pocket is generally larger and can thus accommodate nucleotides with certain modifications. TLS polymerases are characterized by low fidelity, lack of a 3' to 5' exonuclease proofreading activity and poor processivity, as they incorporate only a very limited number of nucleotides.

Arrest of replication at a lesion causes uncoupling of the helicase from the replication fork and ssDNA accumulation, which in turn leads to monoubiquitylation of PCNA at lysine 164 by the E2 ubiquitin-conjugating enzyme RAD6 together with the E3 ubiquitin ligase RAD18 [31, 202]. Monoubiqutylation of PCNA serves as a signal to recruit TLS polymerases via direct interactions with the ubiquitin-binding motif (UBM) or ubiquitin-binding zinc (UBZ) domains of Y-polymerase members [203, 204]. A number of different TLS polymerases with different substrate specificities have been identified. Polη is required for bypass of UV-induced cyclobutane pyrimidine dimers [205], while Polβ and Polλ participate in the accurate bypass of the oxidative DNA lesion 8-oxoguanine.

Stabilization of replication forks

Stalled replication forks can be stable for several hours *in vivo* [206, 207], indicating that there are stabilizing factors, preventing fork collapse.

The ATR/CHK1-dependent S-phase checkpoint control is induced upon RPA binding to ssDNA and prevents late origin firing [208]. One theory of how the checkpoint stabilizes replication forks suggests that cell cycle retardation prevents continued helicase unwinding [209]. Another, more accepted, theory assumes that it regulates HR-mediated fork remodeling for which several models have been proposed. These are described here briefly [74, 112].

Template switching at a fork stalling lesion involves homology search and invasion of the sister chromatid in order to find an unperturbed template to continue DNA synthesis. Behind the lesion, the invading strand will flip back to its original template strand. Another model suggests fork reversal, characterized by regression of stalled forks and re-annealing of the parental strands, to stabilize stalled forks. The two daughter strands will be displaced and annealed creating a four-way junction termed 'chicken-foot'. New synthesis initiated at the daughter strands and branch migration or formation of a double HJ might be required for resolution of chicken-foot structures. Helicases, especially of the RecQ family, have been suggested to be involved in fork stabilization and remodeling [210].

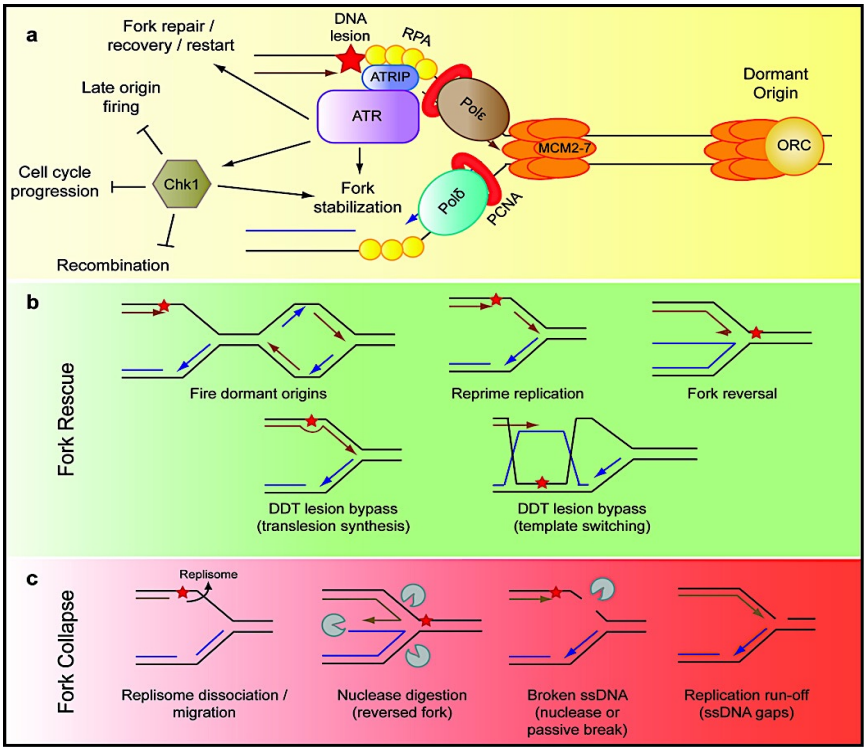


Fig.12: Mechanisms of replication fork restart and collapse. a) At a stalled replication fork ATR and its binding partner ATRIP are activated and ATR initiates a signaling cascade that is primarily mediated through the effector kinase Chk1. Chk1 activation promotes fork stabilization and restart, while inhibiting cell cycle progression until replication is completed. **b)** Replication forks that are stalled due to DNA lesions (red star) can restart replication either by firing dormant origins, repriming replication behind the lesion, reversing the fork or activating DNA damage tolerance pathways. Key intermediates are illustrated. **c)** If stalled forks are not properly stabilized, or persist for longer periods of time, replication forks may collapse. Several possibilities for replication fork collapse are presented here, including dissociation of components of the replisome, nucleolytic cleavage of a reversed or stalled fork (middle panels) by endonucleases or replication run-off (modified from Zeman and Cimprich, 2014 [70]).

Replication fork restart

Blocked replication forks still have access to intact replication machinery and synthesis may simply continue following bypass or repair of blocking lesions. It seems that re-priming of replication is a common event on the lagging strand due to the constant availability of Okazaki fragments. Electron microscope imaging has revealed the presence of ssDNA gaps behind proceeding replication forks in both leading and lagging strands upon adduct formation [211,

212]. These findings indicate that strand synthesis may also proceed in a discontinuous fashion on the leading strand, restarting from new primers. Single-strand gaps may later be filled by TLS polymerases or HR, utilizing the sister chromatid as a template, and seems to be regulated by PCNA ubiquitination in yeast [204].

Cells deprived of essential replication factors promote HR by MRE11-mediated end resection, as re-priming is not an option in this case [213, 214]. HR factors RAD51 and XRCC3 have been demonstrated to be involved in replication fork restart upon short treatment with low dose HU [215].

CANCER CELL METABOLISM

Cancer cells are dependent on increased metabolic rates in order to provide energy to support the rapid proliferation and growth rates associated with malignant transformation. Deregulated cellular metabolism is thus a hallmark of cancer, causing increased metabolic stress and an accumulation of potentially toxic metabolic intermediates. Already in the early 1900s Otto Warburg observed that cancer cells preferably use glycolysis and subsequent anaerobic lactic acid fermentation for energy production, instead of oxidative phosphorylation that requires molecular oxygen. This phenomenon was is thus termed 'Warburg effect'. However, the exact causes for this metabolic switch are still not fully elucidated. Hypoxic conditions in solid tumors could create an environment where glycolysis is a necessary energy supplier. But even in conditions were oxygen is plentiful, glycolytic rates can be up to 200-fold higher in cancer cells, compared to normal tissue. Another advantage of using glycolysis instead of mitochondrial oxidative phosphorylation is the decreased risk of inducing oxidative stress through production of reactive oxygen species (ROS), which are generated as a consequence of electron leakage from the respiratory chain. In order to adapt to their altered redox state, cancer cells might thus switch their metabolism to glycolysis to minimize the endogenous production of toxic oxygen radicals. Glycolysis further provides useful intermediate metabolites that are necessary for nucleoside and amino acid production, which are required for proper replication and proliferation of fast-growing cells [9].

Nevertheless, elevated oxidative stress was observed in cancer cells, which demands adaptation to avoid adverse effects, such as oxidative damage to proteins, lipids and, most importantly, the DNA. Disturbing the cellular redox and ROS balance has therefore been shown to be a promising strategy for novel and more selective anti-cancer treatments due to the increased dependence of those cells on the ROS stress response [8]. Naturally, this includes protection from oxidative DNA injury by repair and prevention of this sort of damage.

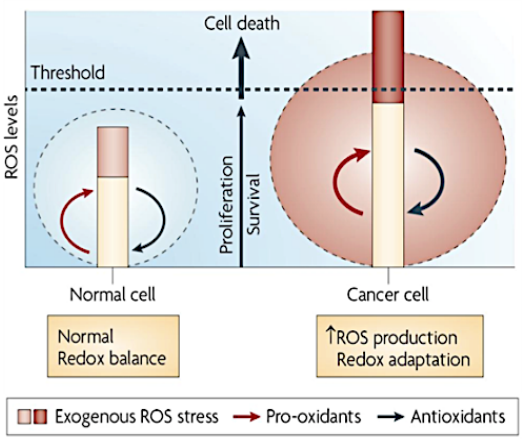


Fig.13: Enhanced ROS production and disturbed redox balance in cancer cells. Due to metabolic abnormalities and oncogene activation, cancer cells exhibit increased production of ROS, which brings them closer to a toxic threshold of tolerable oxidative stress compared to normal cells. In order to cope with increased oxidative stress, cancer cells have to adapt, which they do by shifting their redox dynamics by upregulating their antioxidant capacity. Cancer cells are thus more dependent on the antioxidant system and more vulnerable to further insult by ROS. This phenotype may be exploited to constitute novel anti-cancer therapy (from Trachootham *et al.*, 2009 [9]).

Oxidative stress and disease

Reactive oxygen species are oxygen containing reactive chemical species with an unpaired electron, such as superoxide anions (O_2^-), peroxide (O_2^{2-}), nitric oxide (NO), hydroxyl radicals (OH) or hydrogen peroxide (H_2O_2). They are essential for biological functions by regulating signal transduction, host defense, neurotransmission and vasodilatation [216]. However, because free oxygen radicals are highly reactive with biomolecules, including proteins, lipids and the DNA, they also constitute a major threat to cellular integrity. H_2O_2 has been proposed to play an important role in disease, since it easily diffuses through biological membranes [217].

ROS are produced by environmental mutagens such as ionizing radiation, ultraviolet light, chemotherapeutic drugs or heavy metals, but also arise from normal cellular metabolism, as mentioned earlier. Electron leakage from the mitochondrial respiratory chain, β-oxidation in peroxisomes, inflammation, prostaglandin synthesis and cytochrome P450 detoxification reactions are just a few examples of endogenous processes generating oxygen radicals [218, 219]. Aerobic organisms rely on oxidative phosphorylation to produce energy in the form of ATP. During this process, an electron is transferred to molecular oxygen, transiently producing superoxide anion radicals. The cytochrome oxidase complex in the mitochondrial membrane then ensures detoxification of the reactive oxygen radical by pairing it with two hydrogen atoms, generating a water molecule. However, approximately 1 - 5 % of the electrons escape from the electron transport chain complexes and lead to the formation of intracellular ROS [220, 221]. The exceptionally high growth and metabolic rates of cancer cells correlate with elevated production of oxygen radicals and have been associated with increased levels of oxidative stress [9].

A variety of defense mechanisms have evolved to combat the harmful effects of intracellular ROS. However, despite the activity of detoxifying enzymes and low molecular weight antioxidants, oxidative DNA lesions occur quite frequently [222]. They represent a major threat not only causing mutagenesis and carcinogenesis, but also neurodegenerative disease and ageing [223-225]. Increased ROS production is frequently observed in cancer cells at an advanced disease stage, caused by an activation of oncogenes (such as Ras, Bcr-Abl and c-Myc), aberrant metabolism, mitochondrial dysfunction and loss of p53 activity [226, 227]. Genetic instability thus drives increased ROS generation, which in turn causes further mutations. This 'vicious circle' is self-potentiating and promotes cancer development [228].

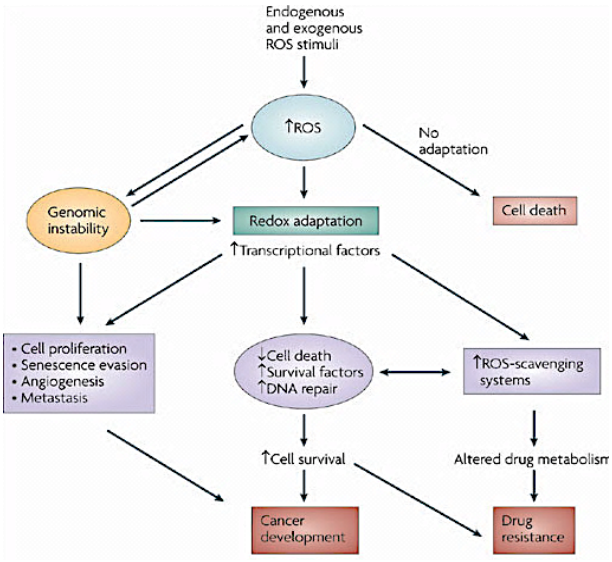


Fig.14: Oxidative stress and its consequences for tumor development. Excessive intracellular ROS accumulation can induce lethal damage in cells that have inadequate stress response or adaptation mechanisms. Persistent oxidative stress may induce an adaptive response in cancer cells, such as activation of redox-sensitive transcription factors (NF-kB and Nrf2) promoting expression of ROS-scavenging enzymes (superoxide dismutase and glutathione), elevated levels of pro-survival factors (BCL2 and MCL1), and inhibition of cell death factors (caspases). DNA mutations induced by RPS promote genomic instability, which provides an additional mechanism for stress adaptation. These events enable survival of cells with high levels of ROS (from Trachootham *et al.*, 2009 [9]).

Antioxidants

Cancer cells have the ability to acquire adaptive mechanisms to counteract the toxic effects of elevated levels of ROS through increased genomic instability. High selective pressure enriches the population of cells that are capable of stress adaptation, for instance by induction of ROS-scavenging systems, promotion of cell-survival or DNA repair pathways [228].

The three major groups of enzymatic antioxidants are superoxide dismutases (SOD), catalases and peroxidases. SOD1 and SOD2 initiate detoxification of superoxide anions into less toxic H_2O_2 and O_2 [229]. H_2O_2 is further detoxified to water by catalases, which are localized in peroxisomes and cytosol. Similarly, glutathione peroxidases are able to reduce free H_2O_2 to water via oxidation of a selenocysteine residue of two reduced monomeric glutathiones (GSH) by H_2O_2, leading to the production of a glutathione disulfide (GS-SG) and two water molecules [229].

Additionally to those enzymatic ROS-detoxifiers, a number of non-enzymatic antioxidants exist, including vitamins (*e.g.* A, C, E), organo-sulfur components (*e.g.* glutathione), coenzyme Q10, enzyme-bound minerals (*e.g.* zinc, selenium), carotenoids, nitrogen compounds (*e.g.* uric acid), phenolic acids and flavonoids [230].

Redox adaptation also involves activation of redox-sensitive transcription factors, such as NF-κB, Nrf2, c-Jun and HIF-1, which among others drive expression of the antioxidants SOD, catalase, thioredoxin and glutathione (GSH). Other transcription factors sensitive to redox imbalances induce the expression of cell-survival molecules, such as anti-apoptotic BCL2 family members and proteins involved in the AKT survival pathway.

Due to the elevated ROS production and altered redox states, cancer cells are likely in a state close to the cytotoxic threshold of oxidative stress. They are therefore more dependent on cellular antioxidants and oxidative stress-evading systems than normal cells and consequently more vulnerable to further ROS insult. Exploiting this phenotype, which is shared among most

cancers, to induce the preferential death of cancer cell was proposed already ten years ago as an anti-cancer strategy and shown to be feasible in experimental systems [8, 231, 232]. However, in order to achieve therapeutic selectivity and overcome drug resistance, it is important to induce oxidative stress and simultaneously abrogate the redox adaptation mechanisms of cancer cells [228].

Oxidative DNA damage

ROS that are able to come into close proximity of DNA can cause damage, due to the high reactivity of these molecules [233, 234]. Especially the electron-rich double bonds in DNA bases (*e.g.* between N7-C8 of purines or C5-C6 of pyrimidines) and their labile hydrogens are vulnerable to radical attack. Guanine has the lowest redox potential of the four DNA bases and is thus particularly susceptible to oxidation [126, 235], generating the main products 7,8-dihydro-8-oxo-guanine (8-oxoguanine, G^0) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapy-G).

In addition to direct oxidation of the DNA bases, oxidative DNA lesions can arise from damaged DNA precursors (2'deoxyribonucleotides) that are incorporated into the DNA during replication. It has recently been suggested that the nucleotide pool is a significant target for oxidants, since it is not protected by the tight chromatin structure like genomic DNA. 8-hydroxy-2'-deoxyguanosine-5'-triphosphate (8-oxo-dGTP) is only one of more than 20 oxidized nucleotides that have been identified so far and it was shown that the relative fractions of 8-oxo-dG and 2-oxo-dA are greater in the free dNTP pool compared to duplex DNA [236]. Thus, nucleotide pool sanitization and BER activity are both important to keep the number of oxidized bases in the genome minimal to prevent mutagenesis, senescence and cell death [237]. In addition to DNA lesions, damaged nucleotides might interfere with various cellular processes, such as signal transduction or metabolism, in which ATP or GTP function as essential mediators or co-factors and therefore exert a certain degree of cytotoxicity themselves [216].

The concentration of 8-oxo-dGTP in the mitochondrial nucleotide pool has been reported to be 1 – 10 %, relative to unmodified dGTP [238] and the actual concentration has been estimated to be below 0.34 μM. Its high mutagenicity emphasizes the importance of nucleotide pool sanitization to prevent carcinogenesis caused by oxidized DNA precursors. However, if altered nucleotides escape degradation and become misincorporated into DNA during replication, the base excision repair pathway serves as an additional line of defense to avoid mutagenesis.

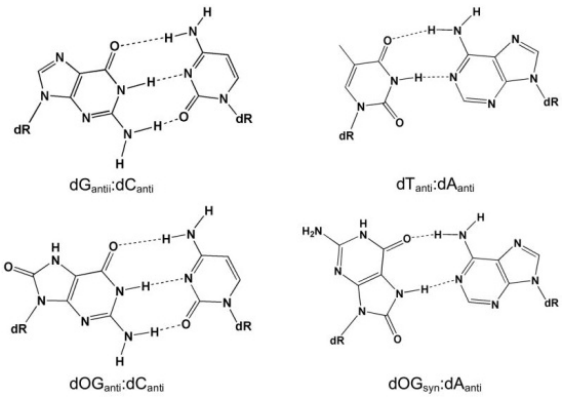


Fig.15: Structure of base-pairs containing guanine (dG) and 8-oxoguanine (dOG). G normally base pairs with C, while T base pairs with A. 8-oxoguanine is generated by the addition of an oxo-group at C8 and a NH-group at N7 of G, facilitating base-pairing with both C (*anti*-configuration) and A (*syn*-configuration) (from David *et al.*, 2007 [126]).

8-oxoguanine

8-oxoguanine (G^o) is the best studied oxidative DNA lesion because of its high abundance, stability and mutagenicity. In normal cells roughly 10^3 lesions arise per cell per day under steady state conditions. This number of lesions can be two orders of magnitude higher in cancer cells and also serves as a good biomarker for oxidative stress [217, 239, 240].

G^o can base pair not only with cytosine, but also with adenine when it adopts a *syn* conformation. This Hoogsteen base pairing of G^o(*syn*)/A(*anti*) is mediated by a hydrogen bridge bond between the hydrogen atom on N7 and the oxo-group on C6 of the purine and specifically induces A:T → C:G transversions [241]. Replicative polymerases efficiently bypass G^o and preferentially incorporate A opposite them [242-246]. Base pairing of G^o(*anti*) with C leads to distortions in the template strand and also in the polymerase. This prevents further interactions of the next template base with the pre-insertion site of the polymerase, thus creating problems for the elongation from a G^o/C pair [245]. The geometry of a G^o(*syn*)/A(*anti*) mispair in the minor groove mimics a T/A base pair without causing distortions. It is therefore not recognized by the proofreading activity of the polymerase and easily elongated. Nevertheless, bypass of G^o was shown to be 80% accurate *in vivo* [242]. A polymerase switch from replicative TLS polymerases Polλ or Polη was shown to mediate correct bypass of G^o. In the presence of PCNA and RPA, Polλ incorporates C with 1200-fold higher efficiency than A and polη with 68-fold higher efficiency [247-250]. However, some G^o(*syn*)/A(*anti*) mispairs are formed in genomic DNA anyways and the misincorporated adenine is excised by the DNA glycosylase MYH to prevent G:C → T:A transversions. OGG1 on the other hand is a glycosylase that removes G^o that is base paired with a cytosine, thus preventing T:A → G:C transversions.

Crucial for efficient sensing and removal of oxidative damage in nuclear and mitochondrial DNA is also the tumor suppressor p53. Loss of functional p53 is associated with increased ROS, a disturbed redox balance and increased mutagenesis, which correlates with tumor aggressiveness and poor prognosis [251, 252].

Damage prevention and base excision repair of 8-oxoguanine

As mentioned earlier, base excision repair (BER) is involved in the repair of oxidative DNA lesions, such as 8-oxoguanine (G^o). As a first line of defense, the hydrolase MTH1 prevents incorporation of the oxidized nucleotide precursor 8-oxo-dGTP into the DNA during replication by its degradation to 8-oxo-dGMP [253]. Oxidized guanines in the DNA that are paired with C are recognized by the glycosylase OGG1, followed by short-patch BER with Polβ [254]. MYH initiates repair of G^o/A mispairs that are formed when G^os are not repaired before another round of replication and A is misincorporated opposite G^o by a polymerase. Upon excision of the adenine and further processing by BER, the original G^o/C mispair is regenerated [255], which now serves as a substrate for OGG1 again. Polλ together with PCNA and RPA were shown to accurately participate in MYH-initiated repair *via* long-patch BER [255, 256].

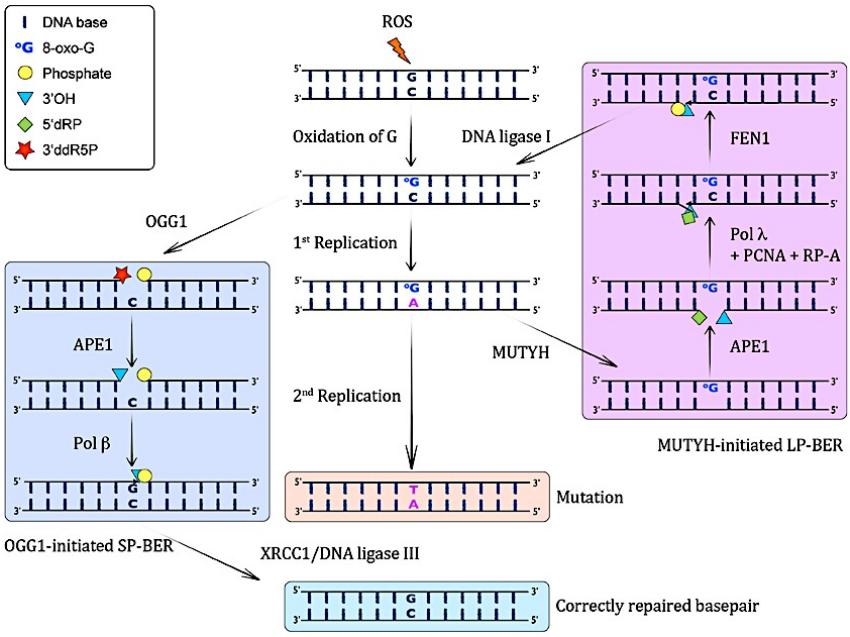


Fig.16: MYH-initiated BER of A:G⁰ lesions. ROS can attack guanines in the DNA, leading to the formation of C:G⁰ base pairs. **Left:** OGG1 excises the G⁰ and uses its lyase activity to incise the AP-site by β-elimination, which gives rise to a 3′ddR5P and a 5′P group. APE1 removes the 3′ sugar phosphate, generating a 1 nucleotide gap with a 3′OH and a 5′P. Polβ then inserts a G opposite the templating C in this SP-BER pathway. XRCC1/DNA ligase I ligate the DNA to restore an intact, correctly base-paired DNA. **Middle:** If C:G⁰ base pairs are not recognized prior to S-phase by OGG1, or they arise through oxidation during S-phase, the replicative polymerases will often mis-incorporate an A opposite G⁰, giving rise to A: G⁰ mispairs, which will lead to a CG→AT transversions, if not repaired before another round of replication. **Right:** MYH (MUTYH) recognizes A:G⁰ base pairs and excises the wrong A. The resulting AP site is further processed by APE1, generating a 1 nt gap with 3′OH and 5′dRP moieties. Polλ incorporates the correct C opposite G⁰ in collaboration with the cofactors PCNA and RPA and FEN1 cleaves the 5′ flap. Finally, DNA ligase I seals the gap to yield an intact C:G⁰ containing double-stranded DNA, which is is then again substrate for OGG1-mediated removal of the G⁰ (modified from Markkanen *et al.*, 2013 [257]).

OGG1

OGG1 is a bifunctional glycosylase with an AP lyase activity that allows generation of DNA nicks *via* β-elimination. However, the intrinsic lyase activity of OGG1 is rather weak (half of the glycosylase activity and therefore rate limiting) and can be stimulated by the addition of APE1 [128, 258]. Human OGG1 represents the functional analog of the bacterial Fpg enzyme and is responsible for the excision of G⁰ opposite C as well as the removal of other oxidized pyrimidines or ring-fragmented purines, such as fapy-G [259, 260].

While OGG1 knock-out in *S. cerevisiae* leads to mutagenesis in the form of G to T transversions, OGG1 null mice have only mildly increased mutation rates and do not develop malignancies [261, 262]. This may be due to the activity of MYH, acting as a 'backup' pathway to avoid mutations. However, MYH activity alone does not eliminate G⁰ in the genome, it is thus not surprising to find elevated levels of G⁰ in the DNA of OGG1 knock-out mice.

Mutations in the human OGG1 gene were identified in lung and kidney cancer; however, it is still unknown whether they contributed to cancer development [263-265].

MYH

As guardian of the genome, MYH prevents the persistence of G^{O}/A mispairs and therefore mutagenesis. Indeed, the levels of G^{O} were increased in cells derived from MYH knock-out mice [266] and enhanced spontaneous mutagenesis was observed in mouse embryonic fibroblasts (MEFs) [256, 267].

Mutations in *MYH* have been shown to be associated with a novel cancer syndrome named MYH-associated polyposis (MAP) [268]. Patients suffering from this disease show multiple colorectal adenomas and carcinomas, which display high proportions of somatic G:C to T:A transversions in their *APC* gene. This mutation footprint is characteristic for deficiency in mutY, the homolog of human *MYH* in *E. coli*. Heterozygous *MYH* missense mutations Tyr179Cys and Gly396Asp (referring to the longest MYH transcript NM_00128425.1) were identified in these patients and it was shown that corresponding mutations in *E. coli* mutY caused decreased activity of those mutants on G^{O}/A and G/A substrates [268]. Collectively, these results indicate that mutations in *MYH* lead to the G to T transversion signature in *APC*, which in turn caused a cancer phenotype that is similar to inherited familial adenomatous polyposis (FAP), a cancer caused by inherited *APC* gene mutations.

The above-mentioned missense variants Tyr179Cys and Gly396Asp include residues, involved in G^{O} recognition [269, 270]. Other variants identified in MAP patients all showed defects in G^{O}/A repair [271-275]. Germline mutations in *MYH* are inherited in an autosomal recessive manner and cause predominantly colorectal polyposis, possibly due to the exposure of the large bowel to high levels of oxidative stress. The *APC* gene is especially susceptible to G to T transversions that predominantly affect GAA sites in MAP patients [268] due to their high abundance in *APC*, which contains 211. Furthermore, G to T mutations in GAA sites generate stop codons and therefore frequently lead to the inactivation of *APC* in the absence of MYH. Since defects in MYH ultimately cause *APC* mutations that are the underlying cause for the polyposis phenotype FAP, both *MYH* and *APC* genes need to be sequenced in order to provide the right prognosis and distinguish FAP from MAP.

Posttranslational modifications were shown to affect the function of MYH. Shrimp alkaline phosphatase (SAP) treatment of native MYH present in human cell extracts decreased the activity of the protein, indicating that phosphorylation is crucial for proper MYH function [276]. Similarly, human colorectal cancer cell extracts lacking *MYH* mutations but displaying defective G^{O}/A repair showed enhanced repair efficiency upon treatment with protein kinase C or A (PKC or PKA) as well as casein kinase II [277]. Ser524 is located in the G^{O} recognition domain within the PCNA binding region of MYH and can be phosphorylated [278]. In addition to phosphorylation, MYH can undergo ubiquitylation by the E3 ligase Mule in between amino acids 475 and 535 [279].

MTH1

As mentioned earlier, free nucleotides are highly susceptible to oxidation by ROS, which underlines the necessity to sanitize the precursor pool to avoid incorporation of damaged nucleotides into DNA during replication. Several enzymes belonging to the **Nu**cleoside **Di**phosphatase linked to another moiety **X** (NUDIX) family are specialized to degrade oxidized

nucleotides and control the intracellular levels of other potentially toxic metabolic intermediates and signaling compounds. They were thus described as 'housecleaning' enzymes [280]. The free nucleotide pool is especially susceptible to oxidation, as it is not protected by the complex chromatin structure like genomic DNA [281]. In order to avoid spontaneous mutagenesis, prevention of DNA damage, through elimination of damaged nucleotides, may therefore be even more important and efficient than DNA repair itself.

The first mammalian Nudix family member was named MutT homolog 1 (MTH1) because of its functional similarity to the *E. coli* MutT protein. MutT degrades the oxidized purine nucleotide triphosphates 8-oxo-dGTP, 2-OH-dATP and 8-oxo-dATP to their monophosphates and PPi, preventing their incorporation into DNA [253] [236]. MutT-defective *E. coli* strains show 100 to 10000-fold increase in spontaneous G:C to T:A transversions, highlighting the essential role in removal of oxidized dATPs [282]. The actions of OGG1 and MYH, as well as their bacterial homologs, may attribute for the lack of G:C to T:A transversions induced by G^0. The degradation product of MutT, 8-oxo-dGMP, is not suitable for DNA synthesis, because the cellular guanylate kinase cannot utilize 8-oxo-dG-containing nucleotides as substrates [283].

In human cells, seven different MTH1 splice variants were identified that encode mainly an 18 kDa transcript, present predominantly in the cytoplasm and mitochondria. They display similar enzymatic activity towards 8-oxo-(d)GTP, 8-oxo-(d)ATP and 2-oxo-(d)ATP, which has been shown to reduce H_2O_2-induced mitochondrial dysfunction and cell death [216]. Furthermore, Rai *et al.* reported that impaired MTH1 expression induces cellular senescence, mainly through the p53 tumor suppressor pathway and defects in DNA replication [236]. MTH1-null mice show a predisposition to develop tumors in lungs, liver and stomach and cells from these animals are sensitive to H_2O_2 treatment [284]. Furthermore, increased MTH1 expression was observed in several tumors (kidney, lung and brain) and a polymorphic MTH1 variant (V83M) has been associated with increased frequency of stomach cancer [285]. Together, these observations indicate a crucial role of MTH1 in preventing spontaneous mutagenesis and tumorigenesis caused by oxygen-induced DNA damage [286].

In 2014, a novel MTH1 inhibitor has been developed that seems to selectively eradicate cancer cells, while leaving normal cells unaffected [8, 287]. Inhibition of MTH1 was suggested as a potential therapeutic approach to selectively target a broad range of tumors that rely on clearance of oxidized nucleotide precursors to prevent oxidative DNA damage, a concept referred to as 'cancer phenotypic lethality'. MTH1 was described as a 'non-oncogene addiction' target, because it is not a typical oncogene, but indispensable for cancer cell survival due to the necessity of cancer cells to adapt to enhanced intracellular oxidative stress and redox imbalances. The applicability of this approach in the clinic remains to be tested in the future.

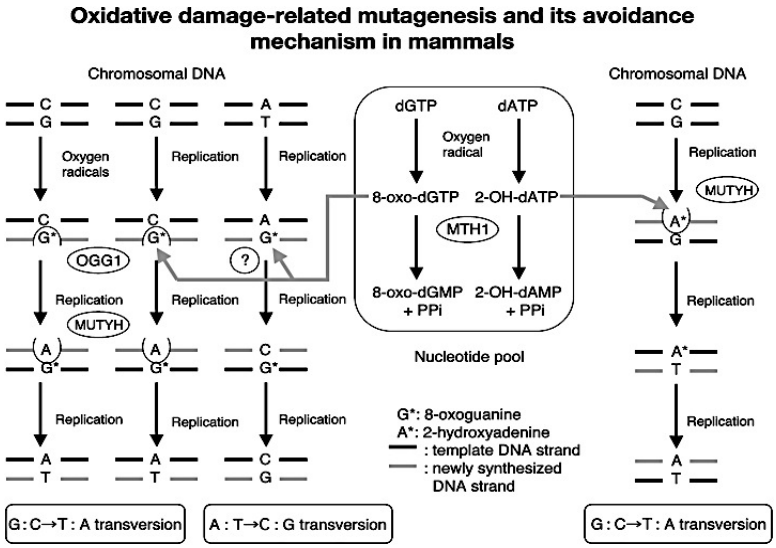


Fig.17: Mutagenesis induced by oxidative DNA damage and its repair mechanisms in mammalian cells. 8-oxo-dG and 2-oxo-dA can form relatively stable base pairs with A or G, respectively. This can lead to A:T → C:G and G:C → T:A transversion mutations. MTH1 effectively hydrolyzes 2-OH-dATP and 8-oxo-dGTP from the nucleotide precursor pool. The glycosylase OGG1 excises 8-oxo-dG and 2-oxo-dA directly from the DNA, while MYH/MUTYH removes A that has been incorporated opposite 8-oxo-dG in the template (from Tsuzuki *et al.*, 2007 [286]).

Other Nudix hydrolase family members

Besides the above-mentioned MTH1, 21 other hydrolases of the Nudix family have been identified with different substrate specificities. MTH2 is also an 8-oxo-dGTPase, which is less selective for oxidized nucleotides than MTH1 and its expression in *mutT*-deficient *E. coli* significantly reduces the spontaneous mutation frequency [288]. In contrast to MTH1 and MTH2, NUDT5 is a Nudix enzyme that preferentially hydrolyzes the oxidized guanine diphosphate 8-oxo-dGDP [289]. 8-oxo-dGTP-induced mutation rates were shown to be significantly increased upon NUDT5 depletion [241] and expression of NUDT5 in *mutT*-deficient *E. coli* mutant cells was sufficient to decrease the spontaneous mutation frequency to normal levels [289].

The fact that 8-oxo-dGTP and 8-oxo-dGDP are interconvertible within the cell (through the actions of the nucleotide diphosphate kinase and the nucleotide triphosphatase) [289] explains the necessity to remove both oxidized nucleotides to efficiently eliminate all potentially harmful DNA precursors from the cell. The involvement of several proteins in clearance of oxidized nucleotides underscores the fundamental importance of avoiding oxidative DNA damage in eukaryotic cells. Furthermore, they prevent transcriptional errors and mistranslation of proteins by detoxifying the precursor pool to avoid incorporation of modified nucleotides into the RNA transcript [290].

The catalytic reaction by NUDIX proteins is dependent on the conserved 23-amino acid motif (Nudix box), Gx_5Ex_5[UA]xREx_2EExGU (U representing an aliphatic, hydrophobic residue) that is located in a loop-helix-loop structure. Conserved Glu residues in the core of the motif, REx_2EE, are essential for binding of divalent cations (in most cases Mg^{2+}) that are required for the enzymatic activity.

Some NUDIX family members are known to degrade ADP-sugars, such as ADP-Ribose. Free ADPR mono- and polymers constitute a major threat to cellular integrity due to their reactivity with proteins and the involvement in Ca^{2+}-signaling, and their clearance is important for balanced metabolism.

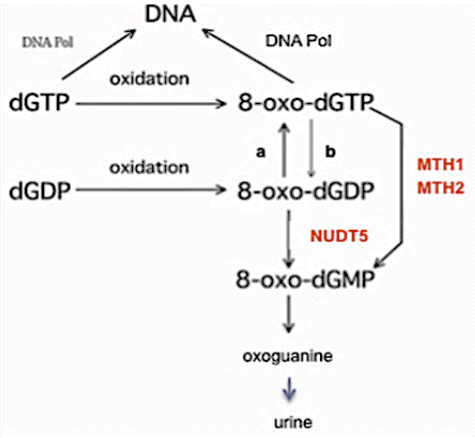


Fig.18: Schematic presentation of the degradation of deoxyribonucleotides containing 8-oxo-dG from the nucleotide precursor pool. 8-oxo-dGTP and 8-oxo-dGDP are interconvertible in the cell by the activity of the nucleoside diphosphate kinase (a) and nucleoside triphosphatase (b). Misincorporation of 8-oxo-dGTP into the DNA during replication can lead to spontaneous mutations. MTH1 hydrolyzes 8-oxo-dGTP, while NUDT5 degrades 8-oxo-dGDP to 8-oxo-dGMP, which is not suitable for DNA replication. MTH1 is inhibited by 8-oxo-dGDP, NUDT5 therefore indirectly promotes the activity of MTH1 by degrading its inhibitor (modified from Sanada *et al.*, 2011 [291]).

ADP-RIBOSE METABOLISM

ADP-ribosylation is a posttranslational modification of proteins, which is of central importance for genomic stability [292] and a wide variety of other biological processes, including transcriptional regulation, [293], chromatin dynamics, centromere function [294], telomere length [295], cell cycle regulation and apoptosis [296]. ADP-ribosylation is carried out by ADP-ribosyltransferases and a subclass of sirtuins (writers), sensed by proteins with specific binding modules (readers) and removed by ADP-ribosylhydrolases (erasers) [297].

ADP-Ribosylation (writers)

Eighteen different human enzymes with ADP-ribosylation activity have been identified thus far and a new nomenclature was proposed due to their differential activity in mono- or poly-ADP-ribosylation (MARylation or PARylation): **A**DP-**r**ibosyl**t**ransferase **D**iphteria toxin-like (ARTD) [298, 299]. However, for simplicity I will continue to make use of the old nomenclature for poly(ADP-ribose)polymerases (PARPs) throughout this thesis. MARylated proteins are primarily found outside the nucleus, while PARylation is mostly observed on nuclear proteins, but both act as scaffolds for the recruitment of proteins during complex formation [300].

Amongst poly-ADP-ribosyltransferases (PARP) family members, PARP1 is the most abundant one sharing overlapping functions with the related protein PARP2 in response to DNA damage. Other proteins with the ability to carry out PARylation are the tankyrases (TNKS1/2) involved in signaling, telomere length regulation and vesicle trafficking [301, 302].

Members of the prominent sirtuin (SIRT) family are mono-ADP-ribosyltransferases (MARTs) that transfer mono-ADPR to histones [303, 304] and may be involved in DSB repair and BER [305]. Mono-ADP-ribosylation could regulate DNA repair and cell proliferation by serving as a marker for the severity of the damage and regulating the pathway choice for repair [306]. For further information on enzymatic and non-enzymatic ADP-ribosylation reactions, I would like to refer to comprehensive reviews by Hassa *et al.*, 2006 and Diefenbach and Buerkle, 2005 [306, 307].

NAD^+ metabolism

The oxidized form of nicotinamide adenine dinucleotide (NAD^+) serves as the source for mono-ADP-ribose, the building block for PAR. Hydrolysis of the high-energy bond between the nicotinamide and the ribose moieties of NAD^+ produces free energy (-34.3 kJ/mol), which is in turn used for enzymatic ADP-ribosylation of proteins [306].

NAD^+ is not only required for the formation of (poly-)ADP-ribose, but it is also an essential cofactor for the generation of energy in form of ATP. Hence, excessive PARP1 activation in response to high levels of DNA damage can lead to a severe and irreversible depletion of the NAD^+ pool and induce cell death due to ATP depletion and energy failure [308, 309]. Intracellular NAD^+ levels actually decrease to 10 – 20% of their normal levels within 5 – 15 min upon exposure of cells to high doses of DNA-damaging agents [310]. Efficient recycling of ADPR and maintenance of balanced NAD^+ pools are thus crucial for energy homeostasis and cell viability.

Interactions with ADP-Ribose (readers)

Regulatory functions of ADP-ribosylation are mainly carried out by noncovalent interactions of 'readers' with ADP-ribose-modified proteins via four distinct binding domains. The PAR-binding domain (PBD) is found in many DNA repair proteins and is composed of a 20-amino-acid-long stretch of basic and hydrophobic residues, specifically recognizing long and branched PAR chains. PAR-binding Zinc-Finger Domains (PBZ) contain a zinc ion, coordinated by two cysteine and two histidine residues and are only found in three human proteins involved in DNA break repair (APLF), checkpoint regulation (CHFR) and PAR metabolism (SNM1A). The globular WWE domain is defined by two tryptophan and one glutamate residue and found in E3 ubiquitin ligases as well as some ARTD family members. Macrodomains are highly conserved globular motifs with 130 - 190 amino acids in length and are involved in many different processes (comprehensively reviewed in [297]).

ADP-Ribosylhydrolases (erasers)

(Poly-)ADP-ribosylation is a reversible protein modification that is removed rapidly and efficiently by ADP-ribosylhydrolases, reducing the half-life of PAR to only a few minutes. Poly(ADP-ribose) glycohydrolase (PARG) is the major enzyme responsible for the degradation of PAR chains, forming both free ADPR polymers and monomers [311]. It hydrolyzes the glycosidic ribose-ribose bonds both exo- and endonucleolytically. However, due to steric hindrance PARG is not able to cleave protein-bound ADP-ribose, leaving behind MARylated proteins. Alternative splicing gives rise to several different PARG proteins with different subcellular localizations (nuclear, cytoplasmic and mitochondrial) and length [312]. ADP-ribosylhydrolase family members (ARH1-3) are structurally distinct from PARG and display distinct specificities towards ADP-ribosylated targets; they are found primarily in the cytosolic fraction. Furthermore, macrodomain-containing hydrolases are recruited to DNA damage sites and some of them were

shown to possess a novel glutamate-specific-mono-ADP-ribosylhydrolase activity, removing the last ADP-ribose unit from glutamate residues [297].

A rapid turnover of nuclear PAR, synthesized in response to cellular stress, may consequently lead to the accumulation of intracellular ADPR units. A large increase of free ADPR can be toxic to cells, because it contains reactive aldehyde groups, which mediate non-enzymatic glycation of proteins on N-terminal lysyl amino acid groups and cysteinyl thiols [306]. Glycation and glycoxidation are protein modifications that can cause substantial damage to long-lived proteins and lead to loss of function. It is therefore of great importance to maintain a balanced pool of NAD^+ as well as ADPR mono- and polymers to reduce cellular toxicity induced by these metabolites.

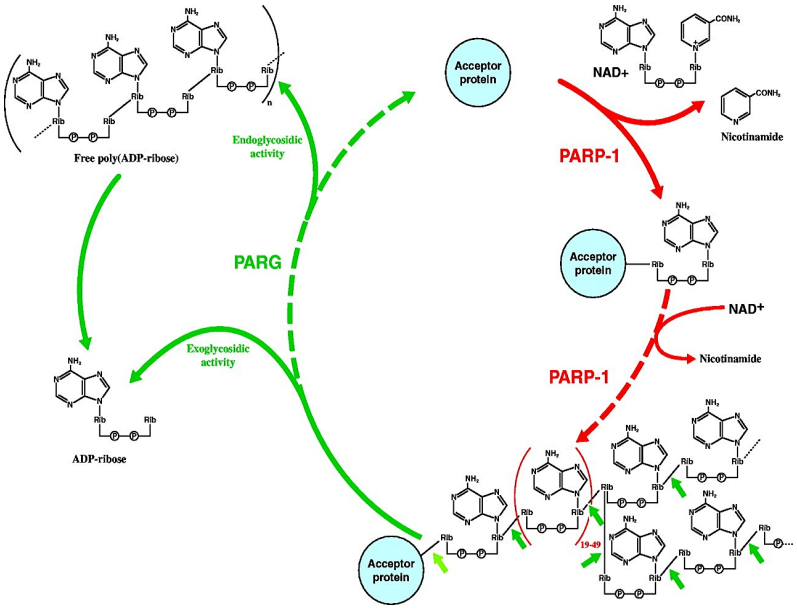


Fig.19: Schematic view of (poly)ADP-ribose metabolism. PARP1 hydrolyzes NAD^+ to attach ADPR units to the target proteins. Negatively charged ADP-ribose polymers have a short half-lives due to the activity of poly(ADP-ribose) glycohydrolase (PARG) that is activated in response to an increasing levels of PAR. PARG cleaves glycosidic bonds between ADP-ribose subunits endo- and exonucleolytically. Free ADPR units can be cleaved by the Nudix hydrolase NUDT5 to Adenosine-mono-phosphate (AMP) and Ribose-5'-Phosphate (R5'P) (from Rouleau *et al.*, 2004 [313]).

PARP1

As mentioned earlier, PARP1 is a poly(ADP-ribose)polymerase that is activated by binding to DNA strand breaks and catalyzes the transfer of ADP-ribose units onto itself and other target proteins [314]. In fact, in response to DNA damage 90% of PAR is synthesized by PARP1. PAR chains serve as a platform to recruit proteins involved in DNA damage recognition and repair and binding of PARP1 to SSB intermediates protects those from further damage. Because of the negative charge of ADP-ribose units, automodification of PARP1 itself leads to its repulsion from the negatively charged DNA, making the interaction of PARP1 with DNA a transient and highly dynamic process.

Despite its role in DNA damage control, PARP1 has multiple other functions, including chromatin remodeling, transcription regulation and the response to a wide range of cellular signals and stresses, such as oxidative, oncogenic, genomic or inflammatory stress [315]. It is a highly abundant nuclear protein, which contains several structural and functional domains. Three zinc finger motifs in the amino-terminus mediate DNA binding, while the central BRCT domain allows automodification and thus interaction with other proteins. The WGR (Tryp-Gly-

Arg) motif seems to be important for inter-domain communications, while the carboxy-terminal catalytic domain is required for NAD^+ binding to the highly conserved signature (Sig) motif and controls PAR catalysis [316]. The PARP regulatory domain (PRD) interacts with the substrate binding site and controls branching of PAR chains [317]. The versatile roles of PARP1 are mainly carried out via posttranslational modification of acceptor proteins to modulate their function, change of interaction partners by automodification and PAR-independent interactions via the BRCT domain. It thus seems to act as a scaffold protein, which can be modified additionally by phosphorylation, acetylation, ubiquitylation and SUMOylation that further broaden its mode of action.

PARP1 in base-excision repair

A role for PARP1 in BER was first anticipated when it became evident that PARP1 knock-out mice, which display no overt phenotype under normal conditions, are sensitive to treatment with monofunctional alkylating agents and γ-irradiation causing DNA damage, which is recognized by BER [318, 319]. Importantly, it was shown that PARP1 is able to bind AP-sites and nicks that are transiently generated by initial base-excision repair steps [320]. These observations challenged the 'passing the baton' model of BER, which described a well-coordinated process, during which single-strand intermediates are passed on from one BER enzyme to the next, avoiding accessibility to other factors. Furthermore, it was shown that PARP1 interacts physically and functionally with key BER factors, such as OGG1, XRCC1 [321, 322], DNA Ligase III and polymerase-β [323].

Due to these results, a 'two-step' BER model was proposed [320], claiming that PARP1 transiently binds and protects nicks generated by the BER glycosylase and APE1 [324, 325]. Activation of PARP1 upon binding to the nick triggers automodification and therefore generates a 'recruiting platform' for XRCC1, Polβ and DNA LigIII at the site of damage. At the same time, excessive PARylation leads to repulsion of PARP1 from the DNA, which allows accessibility of downstream BER enzymes to complete the repair process. PARP1 thus seems to stimulate BER [320, 323, 326] and accelerates SSBR [327], but it is not absolutely required for these repair processes [328]. This is highlighted by the fact that PARP1 knock-out mice display no overt phenotype under normal conditions, while APE1, Polβ and XRCC1 knock-out mice are not viable [329-331]. Furthermore, alkylation-induced repair of SSBs is not affected by PARP1 status [328] and SSB-containing plasmids are repaired with the same efficiency in cell extracts with and without PARP1 [314]. However, it has been proposed that PARP1 activity may be an important 'sensor' for the recognition and accelerated repair of 'direct' SSBs that arise in the context of the complex chromatin environment [139]. Furthermore, recruitment of the important BER scaffold factor XRCC1 to the site of the lesion stimulates repair about 5-fold [332].

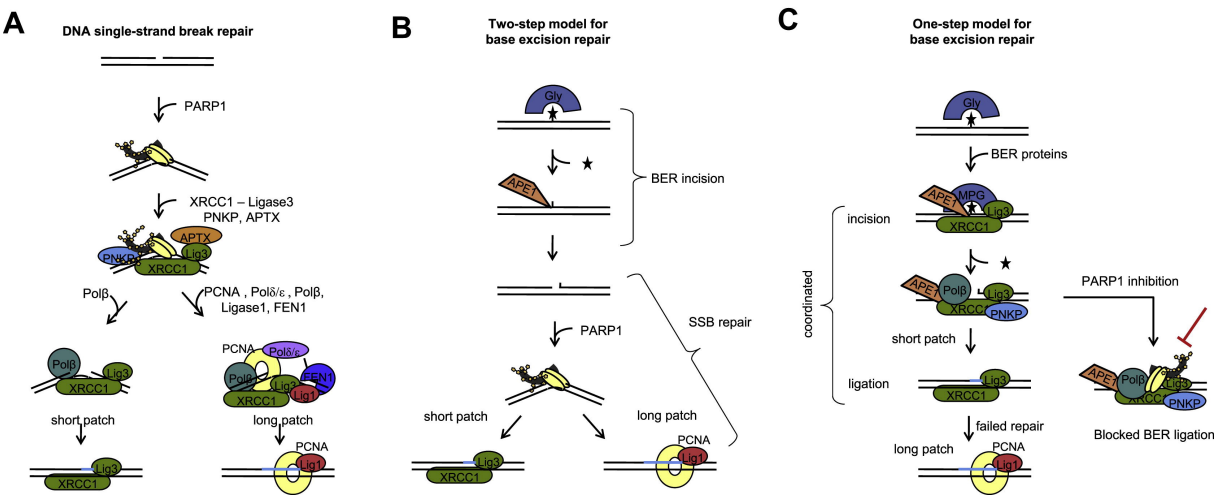


Fig.20: Base-excision repair (BER) is a distinct from DNA single-strand break (SSB) repair in mammalian cells. A) *SSB repair*: PARP1 binds with high affinity to SSBs and is thus amongst the first proteins binding the lesion. In turn it recruits factors involved in end processing and ligation. **B)** *Two-step BER model*: Small base lesions are recognized and excised by different DNA glycosylases (Gly), before incision of the DNA by the AP-endonuclease (APE). SSBs are left unprotected and recognized in a separate process by PARP1 to initiate SSB repair. **C)** *One-step BER model*: The glycosylase removes the damaged base just before APE incision and already interacts with other proteins involved in the early incision step. The half-life of the SSB intermediate is very short and rapidly ligated by short-patch repair, switching to long-patch repair in case of ligation difficulty. PARP1 is not directly involved in BER, but can transiently bind the SSB intermediate. When PARP1 activity is inhibited, it gets trapped on the SSB intermediate preventing ligation (modified from Helleday, 2011 [326]).

PARP1 in double-strand break repair

PARP1 has been implicated in the repair of DSBs, as PARylation is thought to recruit appropriate repair factors to the site of the lesion [4]. Additionally, PARP1 activity is important for resolution of stalled replication forks. It binds to stalled forks and mediates fork reversal as well as recruitment of MRE11 to allow processing and fork restart [213]. Further, PARP1 seems to inhibit toxic NHEJ events at collapsed replication forks [139].

Despite the ability of PARP1 to bind DSBs its role in classical NHEJ is still not clear. PARP1 and KU/DNA-PK physically interact [333] and modify each other by PARylation and phosphorylation, respectively [334, 335]. Furthermore, PARP1 seems to be required for alternative NHEJ, a pathway that takes over in the absence of KU70/80 and independently of DNA-PK and XRCC4-LigIV [263]. Together with XRCC1, PARP1 may promote end-joining (synapsis) of the broken DNA ends and recruitment of LigIII to facilitate final ligation [336].

PARP1 INHIBITION AND SYNTHETIC LETHALITY

The concept of synthetic lethality is based on the redundancy of certain pathways that normally serve as backup pathways for one another and thus both need to be deactivated to induce cell death. The clinical applicability of this approach was nicely shown more than ten years ago, when it became evident that BRCA1- or BRCA2-deficient tumors are specifically killed by PARP inhibitors in the absence of additional exogenous DNA damaging agents [2, 3]. Due to the specific toxicity of this treatment to BRCA mutated cancer cells with deficiency in homologous recombination, side effects should be very much reduced compared to conventional chemotherapeutics. The first PARP inhibitors were NAD^+ analogs that compete with NAD^+ for its binding pocket [337]. The biggest challenges were then to develop inhibitors with better substrate specificity, especially due to the high sequence similarity of the PARP family members.

The mode of action of PARP inhibitors in homologous recombination (HR) deficient cells was proposed to be mediated through the role of PARP1 in single-strand break repair (SSBR). It was shown that PARP1 promotes BER by recruiting downstream repair factors, such as XRCC1, Polβ and LigIII. Upon PARP1 inhibition this pathway is thought to be interrupted, leading to an accumulation of SSBs that collapse into DNA double-strand breaks (DSBs) when they are encountered by the replication machinery. These highly cytotoxic lesions are normally repaired by HR during S- and G2-phases of the cell cycle. However, in the absence of BRCA1 or BRCA2, homologous recombination is impaired and cells undergo apoptosis due to the high loads of DNA damage [2, 3]. Additionally, it was shown that HR-deficient cells treated with PARP inhibitors induce non-homologous end-joining joining (NHEJ) to repair DNA DSBs. This pathway is error prone and can cause genomic instability, which ultimately also leads to cell death [338].

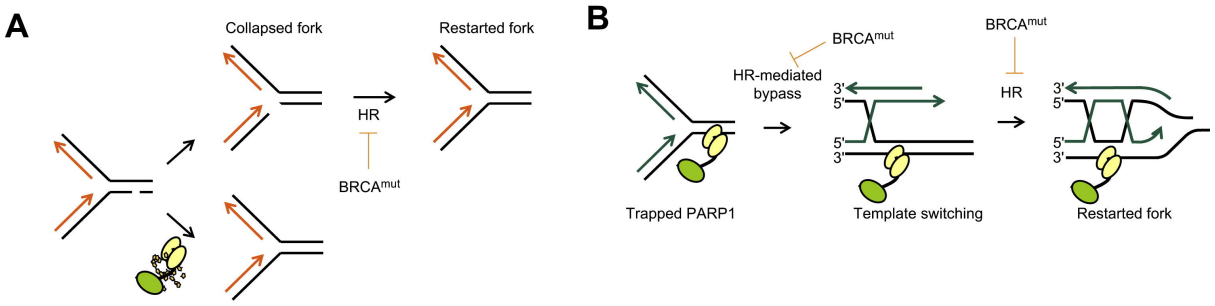


Fig.21: Models for synthetic lethality between PARP and BRCA. A) *SSB replication run-off model*. PARP1 is involved in the repair of SSBs. In the presence of a PARP inhibitor, SSBs may persist and collapse during an encounter with the replication fork into a one-ended DSB. BRCA-defective cancer cells lack HR and are thus unable to repair the resulting DSBs in an accurate manner, leading to toxicity. **B)** *PARP1 trapping model*. PARP inhibitors trap PARP1 onto SSBs that have formed spontaneously or arise as intermediates during BER. PARP1-DNA complexes may present an obstacle to replication that require HR for repair (modified from Helleday, 2011 [326]).

Surprisingly, siRNA-mediated knock-down of PARP1 in BRCA-deficient cells is less toxic compared to PARP1 inhibition in the same cells [2]. Furthermore, depletion of PARP1 does not affect alkylation-induced SSB repair [328] and knock-down of the essential BER factor XRCC1 does not kill BRCA2-deficient ovarian cancer cells [338]. Collectively, these experimental results led to the proposal of the so-called 'trapping model', which suggests that PARP1 inhibition

allows binding of the enzyme to SSBs, but prevents its automodification and thus repulsion from the DNA. PARP1 would therefore be trapped on the SSB and could be converted to even more toxic lesions during replication and other processes [326], which require HR activity for faithful repair. According to the 'trapping model', PARP1 levels in tumor cells would increase the effectiveness of PARP inhibitors, while the original model would state the opposite. A detailed understanding of the exact mechanism will be important for personalized medicine.

It has been proposed that PARP inhibitors may also be effective at killing cells proficient in BRCA1/2, possibly through processes unrelated to DNA repair [339, 340].

PARP1 inhibitors in the clinic

Following the discovery that PARP inhibitors are highly cytotoxic in BRCA-deficient cancer cells [2, 3], first clinical trials were initiated using the PARP inhibitor Olaparib in ovarian cancer patients with inherited BRCA1 or BRCA2 mutations [341]. In 2014 Lynparza™ (containing the PARP inhibitor Olaparib) was finally licensed in the European Union as well as the United States as first-in-class treatment for maintenance monotherapy of patients with platinum-sensitive relapsed BRCA-mutated high-grade serous epithelial ovarian, fallopian tube or primary peritoneal cancers. The most common adverse events associated with Olaparib monotherapy were rather mild and include nausea, vomiting, fatigue and anemia.

In recent years, research has focused on the identification of other gene mutations that are sensitive to PARP inhibitors. Worthwhile to mention here are synthetic lethal interactions with PTEN, a tumor suppressor gene that is frequently mutated in prostate, endometrial, skin and brain tumors and causes HR-deficiency [342, 343]. MRE11-deficiency also causes HR-defects and was shown to induce PARP inhibitor sensitivity in microsatellite unstable colorectal cancer cells [344]. Interestingly, defects in cell cycle regulators such as ATM also leads to PARP inhibitor sensitivity [345]. This is quite interesting, as synthetic lethal interactions with cell cycle regulators in combination with other DNA repair pathways could be envisioned and ATM inhibitors are currently under investigation as novel cancer therapeutics.

PARP inhibitors were also tested in various clinical trials as combination therapy approaches with DNA damage inducing drugs, such as alkylating agents, topoisomerase I inhibitors, platinum drugs, radiation, nucleoside analogs or antimetabolites. The concept behind these treatment modalities is to generate a high amount of DNA lesions that require PARP1 activity and even exceed the repair capacity of HR-competent cells.

PARP inhibitor resistance

A major problem of PARP1 inhibitors and many other drugs is resistance that is often the consequence of the high mutation rate of malignant cells and their ability to adapt to different environmental challenges. Several molecular mechanisms for (adapted) resistance to PARP1 inhibitors were reported in pre-clinical and clinical settings. Elegant studies by Edwards *et al.* identified secondary mutations in BRCA2 that restored the open reading frame through intragenic deletions, which re-activated HR ability in CAPAN1 cells [346]. Later, several studies showed that also loss of important factors involved in NHEJ, such as 53BP1 and REV7, can lead

to PARP inhibitor resistance in HR-deficient cells by partial restoration of functional HR, *e.g.* through alternative loading of Rad51 onto ssDNA and promotion of end resection [182, 183, 347]. Furthermore, inhibition of NHEJ prevents deleterious genomic rearrangements and therefore reduces the toxicity of PARP inhibitors. Another mechanism by which cancer cells achieve drug resistance is through upregulation of multidrug resistance (MDR) transporters, such as P-glycoproteins, that effectively reduce the intracellular availability of xenobiotics [348].

With regard to personalized medicine, it will be crucial to identify further resistance mechanisms to avoid treatment of patients who will likely not respond to the treatment due to specific mutations or gene expression profiles. Novel approaches further aim at overcoming drug resistance to be able to make use of the tremendous therapeutic potential of PARP inhibitors. One strategy is for instance to generate inhibitors that are not targeted by MDR-transporters like AZD2461.

AIMS

A detailed understanding of genome stability and the underlying repair mechanisms that prevent mutagenesis is crucial in the field of oncology. Endogenous DNA damage is emerging as an abundant and important source of DNA lesions, which substantially contribute to the onset and progression of cancer. Furthermore, germline or somatic mutations in DNA repair genes are found in a subset of tumors, leaving them more dependent on backup repair pathways to support cell survival. This concept has been exploited in cancer therapy, in an attempt to find novel synthetic lethal interactions of different DNA repair pathways.

The first aim of this thesis was to further investigate and unravel the basis of a well-known synthetic lethal interaction between PARP inhibitors and homologous recombination (HR) deficiency. PARP inhibitors prevent efficient repair of single-strand breaks (SSBs), which are converted into even more toxic lesions that require HR for accurate repair. We argued that endogenous stress, primarily oxidative DNA damage, contributes to PARP inhibitor sensitivity. Our research focused on one particular lesion, 8-oxo-guanine (G^{O}), that can mispair with adenine (A) to give rise to G^{O}:A mismatches. The base-excision repair pathway, initiated by the DNA glycosylase MYH, addresses these particular lesions and leads to the accumulation of transient SSBs, which potentially trigger PARP activation. We therefore set out to test whether this particular repair pathway contributes to PARP inhibitor activity in HR-defective cells.

Another aspect of this thesis focused on elucidating the role of ATAD5, a putative DNA repair factor, in DNA damage prevention in mammalian cells. We aimed at unraveling its requirement as a proposed PCNA unloader for the maintenance of genome stability and to uncover other functions of ATAD5 in DNA metabolism. Furthermore, we hypothesized that ATAD5 may be involved in DNA replication and therefore carried out a series of DNA fibre experiments to test this experimentally.

RESULTS I

Oxidative DNA damage repair initiated by MYH contributes to PARP inhibitor sensitivity in HR-deficient cells

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Abstract

Homologous recombination (HR)-deficient cells, such as BRCA1- or BRCA2-mutated breast and ovarian cancer cells, are intrinsically sensitive to poly(ADP-ribose)polymerase (PARP) inhibition - a concept that is generally referred to as synthetic lethality (1,2). This hypersensitivity to PARP inhibitors, such as Olaparib, is thought to result from accumulation of DNA single-strand breaks (SSBs), which are converted into deleterious double-strand breaks (DSBs) during encounters with the replication machinery (1,2). HR-defective cells treated with PARP inhibitors rely on the error-prone non-homologous end-joining (NHEJ) pathway for DSB repair, leading to elevated levels of genomic instability and cytotoxicity (3). However, the initial source of DNA lesions that require PARP activity for their repair still remains speculative. We hypothesized that one such source might be SSB-intermediates generated during the processing of endogenous oxidative DNA damage by base excision repair (BER). Particularly, we asked whether processing of potentially mutagenic 8-oxo-guanine (G^0)/A mispairs by the DNA glycosylase MYH could contribute to Olaparib sensitivity in HR-defective cells. Here we show that siRNA-mediated depletion of MYH in BRCA1- or RAD51-depleted cells partially rescues Olaparib sensitivity and DNA strand break accumulation in several different cancer cell lines. Collectively, our results indicate that MYH-induced processing of oxidative DNA damage contributes to PARP inhibitor sensitivity of HR-defective cells.

Introduction

BRCA1- and BRCA2-deficient cells are highly sensitive to treatment with inhibitors of poly(ADP-ribose)polymerase (PARP) (1,2). BRCA1 and BRCA2 are important tumor suppressor genes involved in homologous recombination (HR) repair (reviewed in (4-6)), a pathway required for efficient and accurate repair of DNA double-strand breaks (DSBs). DSBs are highly cytotoxic lesions due to their potential to cause genomic rearrangements and their ability to trigger cell cycle arrest or apoptosis. PARP-1 is an enzyme with a variety of different functions in DNA metabolism, one being its involvement in single-strand break repair (SSBR), a sub-pathway of the base-excision-repair (BER) pathway. PARP-1 accelerates global SSBR to facilitate fast and efficient repair of single-strand breaks (SSBs), which are the most common lesions in cells that can arise from direct attack of the DNA, abortive activity of topoisomerase 1 or as normal intermediates of BER (7). Upon binding to DNA strand breaks, PARP-1 is activated and catalyzes the synthesis of long chains of poly(ADP-ribose) (PAR) on itself and other acceptor proteins (8-10). Using small molecule inhibitors it was shown that global PARP inhibition dramatically slows down DNA strand break repair (11-15).

The mode of action of PARP inhibitors was thus proposed to be attributed to an accumulation of DNA SSBs, which are converted into deleterious DSBs as a result of encounters with the replication machinery (reviewed in (16-20)). HR-deficient cells are incapable of faithful repair of these lesions and rely on the more error-prone non-homologous end-joining (NHEJ) pathway, which may lead to chromosomal rearrangements, genomic instability and cell death. However, the initial sources of SSBs, which require PARP activity in cells that have not been exposed to DNA damaging

agents, still remains a matter of speculation. Intracellular oxidative stress may be one of the main contributors of cellular SSB accumulation, due to the fact that normal cellular metabolism can generate intracellular reactive oxygen species (ROS), which readily attack proteins, lipids and the DNA (21,22). One of the most frequent and well-studied oxidative DNA lesions is 7,8-dihydro-8-oxo-guanine (8-oxo-G or G^o) with a steady-state level of 10^3 lesions per cell in normal tissues (21,23,24). Frequently, adenine is mis-incorporated opposite G^o by replicative polymerases, leading to the formation of G^o:A mispairs, which can give rise to G:C to T:A transversion mutations when not repaired before subsequent rounds of replication (25). These lesions are primarily eliminated by BER, which is initiated by the DNA glycosylases MYH and OGG1 (26). While OGG1 removes G^o paired with C, MYH excises adenines that have mis-paired with G^o in order to allow subsequent repair by OGG1 to restore the correct G:C pair (24). Interestingly, these glycosylases have a different mode of action: OGG1 initiates a short patch BER reaction in which Polβ fills a one-nucleotide gap that is later sealed by the DNA ligase III/XRCC1 complex. MYH, on the other hand, induces long patch BER that is coordinated by proliferating cell nuclear antigen (PCNA) and replication protein A (RPA). Following lesion bypass by Polλ, which preferentially incorporates dCTP opposite G^o (27-29) the flap endonuclease 1 (FEN1) is recruited by PCNA to remove the 5'-single-strand DNA (ssDNA) flap, allowing DNA ligase I to seal the nick (29). The resulting G^o:C pair is, as mentioned earlier, a substrate for OGG1. Both repair events create transient SSBs that may require PARP activity for global BER. This may be important, especially in the context of chromatin, where repair proteins need to be attracted specifically and rapidly to the site of the lesion. PARP1 may thus help to facilitate recruitment of repair factors to sites of DNA damage that are difficult to access in the complex chromatin environment.
Furthermore, cancer cells presumably display elevated levels of oxidative stress, due to oncogene activation, enhanced cell growth and altered metabolism (21). We thus reasoned that processing of DNA lesions induced by endogenous oxidative stress require PARP activity for efficient repair and thus induce sensitivity in an HR-deficient background. Indeed, we show that MYH knock-down partially rescues Olaparib toxicity in BRCA- or Rad51-depleted cells. Furthermore, we observed reduced amounts of SSBs and DSBs when MYH and BRCA1 or MYH and Rad51 were co-depleted, suggesting that processing of potentially mutagenic G^o:A mis-pairs by MYH generates transient SSBs that contribute to PARP inhibitor sensitivity in HR-defective cancer cells.

Material and Methods

Antibodies for Western Blot
Rabbit α-53BP1 (Santa Cruz Biotechnology, 1:1000), mouse α-BRCA1 (Santa Cruz Biotechnology, 1:100), mouse α-CtIP (Santa Cruz Biotechnology, D4, 1:250), mouse α-Lamin B1 (Abcam, ab16048, 1:100), mouse α-MYH (Abcam, ab 55551, 1:333), rabbit α-OGG1 (Abcam, ab124741, 1:10000), rabbit α-Rad51 (Santa Cruz Biotechnology, sc-293, 1:1000), rabbit α-RNaseH2 (GeneTex, GTX85020, 1:1000), mouse α-RPA2 (Calbiochem, 1:50), and rabbit α-pRPA2 (Ser-4/Ser-8, Bethyl Laboratories, 1:500) were used. Horseradish peroxidase (HRP)-conjugated secondary anti-mouse and anti-rabbit antibodies (GE Healthcare) were used at a dilution of 1:5000.

Cell culture
A2780 cells were grown in DMEM (GIBCO) supplemented with 5% Tet-Off FCS, streptomycin/penicillin (100 U/ml), blasticidin and puromycin. HCT116 cells and HCT116 + Chromosome 3 cells were grown in McCoy's medium (GIBCO) supplemented with 10% FCS and streptomycin/penicillin (100 U/ml).

siRNA transfection
Cells were seeded to approximately 30-50% confluency and transfected with 40 pmol siRNA oligonucleotides using Lipofectamine RNAiMAX™ (Invitrogen) according to manufacturer's instructions. The medium was refreshed 20 hours post-transfection and cells re-seeded for 48h after transfection. The following oligonucleotide sequences were applied:
Luciferase (siLuc) 5'-CGUACGCGGAAUACUUCGA-3' (Microsynth, Switzerland),
MYH 5'-UCACAUCAAGCUGACAUAUCAAGUA-3' (Microsynth, Switzerland),
BRCA1 5'-ACCAUACAGCUUCAUAAAUAA-3' (Microsynth, Switzerland),
RAD51 5'-GAGCUUGACAAACUACUUC-3' (Microsynth, Switzerland),
RNaseH2 5'-GGACUUGGAUACUGAUUAU-3' (Microsynth, Switzerland).
OGG1 SMART pool (4968, M-005147-03-0005, Dharmacon) consisting of:
5'-GAUCAAGUAUGGACACUGA-3', 5'-AGAGGUGGCUCAGAAAUUC-3',
5'-GGUUCUGCCUUCUGGACAA-3' and 5'-GGAGCAAAGUCCUGCACAC-3'

CellTiter-Blue® cell viability assay
For viability assays, cells were seeded in triplicates in 96-well plates at a density of 750 (A2780), 1000 (HCT116 and HCT116+3) and 2000 (SUM149PT) per well 48 hours after siRNA transfection. 24 hours after seeding, the cells were treated with the indicated concentrations of Olaparib. Treatment with Olaparib was performed continuously for 72 or 96 hours, as indicated. Cell viability was measured with the CellTiter-Blue® Cell Viability Assay (Promega) according to the manufacturer's instructions. Fluorescence ($560_{Ex}/590_{Em}$ nm), an indicator of metabolic activity, was measured 4-6 hours after addition of CellTiter-Blue® reagent.
Whole cell extracts for Western blot analysis were prepared on the day of the drug treatment, using 2x Laemmli buffer (120 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol).

Immunofluorescence
Cells were cultured on glass coverslips and 24 h post seeding cells were treated with 10μM Olaparib for 24 hours. Subsequently, cells were fixed with 3.7% formaldehyde for 10min at room temperature (RT) and permeabilized using 0.2% (v/v) Triton-X-100 in PBS for 5min at RT. After blocking with 5 mg/ml BSA in PBS for 30min at RT, cells were incubated with the primary antibody diluted in blocking solution for 1.5 h at RT: mouse α-BRCA1 (Santa Cruz; 1:100). Slides were washed with PBS two times 10 min and then incubated for 1 hour at RT with according secondary antibody in blocking solution: alexa-488 conjugated goat anti-mouse IgG (Invitrogen; 1:1000). After washing twice with PBS for 10 min and rinsing the slides once quickly in ddH_2O, coverslips were mounted on Vectashield with 4'6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Images were captured with an Olympus IX81 fluorescence microscope and at least 50 cells were analyzed in each of three independent experiments.

Chromatin-binding Assay
Chromatin fractionation was adapted from the previously described protocol described in (30). Briefly, cells were harvested and incubated in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 1 mg/ml digitonin, Roche complete protease inhibitor cocktail and addition of phosphatase inhibitors: 1 mM $NaVO_3$ and 10 mM NaF) on ice for 12 min. Nuclei were washed twice with PBS and chromatin-bound proteins were extracted using 2x Laemmli buffer (120 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol). Samples were sonicated for 25 seconds at amplitude 72, 50 cycles (Bandelin Sonoplus GM70) and subsequently prepared for western blot analysis.

Alkaline Comet Assay
The CometAssay® kit from Trevigen® was used as described per manufacturer's protocol. Briefly, 48 h after transfection with siRNA cells were re-seeded into 10 cm dishes and exposed to 10 μM Olaparib for 24 h the following day. Cells were re-suspended in ice-cold PBS at a concentration of 2 x 10^5 cells/ml, embedded in molten LMAgarose at a ratio of 1:10 and spread on CometSlides™. The slides were immersed in 4°C Lysis Solution for 30-60 min before exposure to Alkaline Unwinding Solution (pH>13) for 20 min at RT and electrophoresis at 21 V for 30 min. Subsequently, slides were washed twice in dH_2O and once in 70% ethanol, dried at 37°C for 10-15 min and stained with SYBR® Green for 30 min. Images were captured with an Olympus IX81 fluorescence microscope and at least 60 cells were analyzed in each of three independent experiments.

Pulsed Field Gel Electrophoresis
Pulsed field gel electrophoresis was performed as described previously (31). Briefly, cells were transfected with siRNA in 6-well format, re-seeded into 10 cm dishes 48 h after transfection and treated the day after with 10 μM Olaparib for 24h. Cells were then harvested by trypsinization and agarose plugs were generated containing 250000 cells/plug. Quantifications were performed using ImageJ software, and graphs generated using GraphPad Prism.

Growth curves
A2780 cells were transfected with siRNA and 72h after transfection, cells were re-seeded into 12-well plates at a concentration of 20000 cells/well. The following day cells were treated with 10 μM Olaparib for the indicated time points. Cells were then counted and absolute number of cells was calculated.

FACS Analysis
72 hours after siRNA transfection, cells were treated with Olaparib at 10 μM for 24 h. Cells were then collected, washed once with PBS and subsequently fixed by drop-wise addition of ice-cold 80% ethanol. For samples preparation, cells were washed once with PBS and stained with 25 μg/ml propidium iodide (Fluka) supplemented with 100 μg/ml RNaseA (Sigma) for 30min at RT. Samples were analyzed on a Cyan ADP flow cytometer (Beckman Coulter) fitted with Summit software v4.3 (Beckman Coulter).

Results

MYH-depletion partially rescues toxicity of BRCA1-depleted and -deficient cells towards the PARP inhibitor Olaparib
PARP inhibitors are a novel class of anticancer drugs used for the treatment of BRCA1- or BRCA2-deficient breast and ovarian tumors that display a defect in homologous recombination (HR). It is believed that the toxicity of these agents in HR-defective cells is mediated through an accumulation of DNA single strand breaks, which are converted to deleterious double strand breaks as a result of encounters with the replication machinery. In order to investigate the origin of the causal single strand breaks (SSBs), we investigated the involvement of a specific DNA glycosylase MYH, which is crucial for the repair of 8-oxo-guanine (G^0) lesions that are paired with adenine (A) (29). MYH recognizes G^0:A pairs and excises the misincorporated adenine to prevent mutagenesis (29,32,33). Here we show that simultaneous siRNA-mediated knock-down of MYH and BRCA1 partially rescues the sensitivity of A2780 ovarian carcinoma cells to the PARP inhibitor Olaparib, as compared to cells depleted of BRCA1 alone (Fig. 1 A). The knock-down efficiency of BRCA1 and MYH is shown by Western Blot analysis (Fig. 1 B) and

BRCA1-depletion is additionally confirmed by immunofluorescence imaging (Fig. 1 C&D). Notably, BRCA1 forms nuclear foci upon high-dose Olaparib treatment, which underscores the requirement for HR activity in cells exposed to PARP inhibitors.

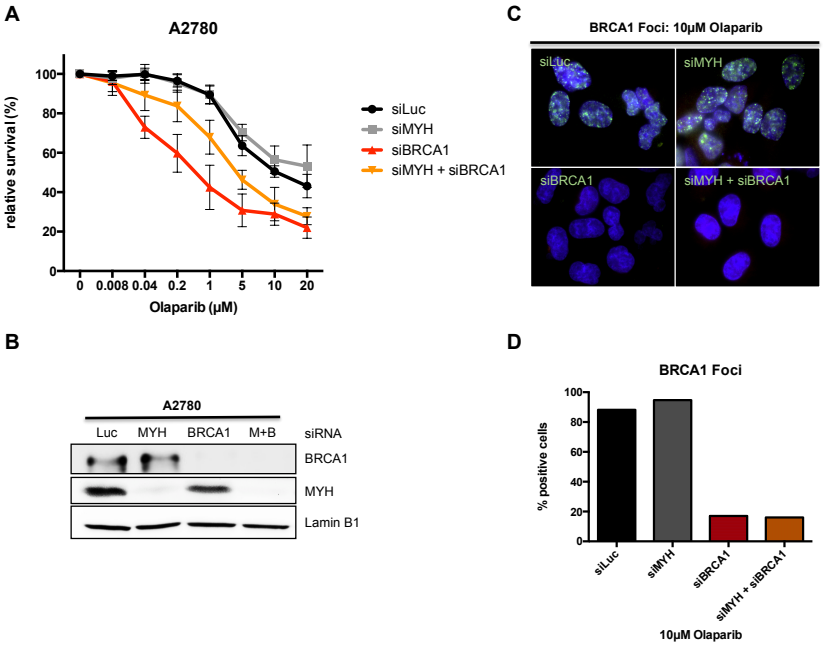

Fig.1: MYH co-depletion partially rescues Olaparib sensitivity of BRCA1-depleted A2780 ovarian carcinoma cells. A) Cell Titer Blue survival assay of A2780 cells transfected with siRNA targeting MYH, BRCA1 or a combination of both treated with increasing doses of Olaparib for 72h. The survival of untreated cells was set to 100%. Error bars represent SD (n = 3). B) Western Blot analysis of BRCA1 and MYH knock-down efficiencies. C) Immunofluorescence imaging using anti-BRCA1 antibody and 4'6-diamidino-2-phenylindole (DAPI) upon treatment of siRNA-transfected cells with 10 μM Olaparib for 24h. D) Quantification of BRCA1 positive cells (> 10 foci per nucleus) depleted of MYH, BRCA1 alone or in combination.

To test whether the observed phenotype is a general one, we depleted BRCA1 and MYH in combination with BRCA1 in several different cancer cell lines (Fig. 2). Similarly to A2780 cells, MYH and BRCA1 co-depletion reduces Olaparib toxicity in mismatch repair-deficient HCT116 colon cancer cells as well as HCT116 cells complemented with part of chromosome 3 (HCT116 + 3) (Fig. 2 A), in which mismatch repair (MMR) is restored (34). It thus appears that the cellular MMR status does not influence the involvement of oxidative DNA damage repair by MYH on PARP inhibitor sensitivity in BRCA1-depleted cells. This is an interesting observation, because it was shown that not only base-excision-repair (BER), but also the MMR machinery can recognize and process oxidative DNA damage (35-37). Notably, MMR-deficient cells (HCT116) reacted in general slightly more sensitive to Olaparib treatment when compared to their MMR-proficient counterparts (HCT116+3). This was already observed previously (38), however, the exact mechanism for this is still unclear. In addition, we were able to recapitulate the MYH-dependent rescue phenotype in U2-OS ovarian sarcoma cells (data not shown). Collectively, these results suggest that MYH-dependent BER of G^0:A lesions contributes to Olaparib sensitivity in a variety of different cancer cell lines that display a defect in BRCA1.

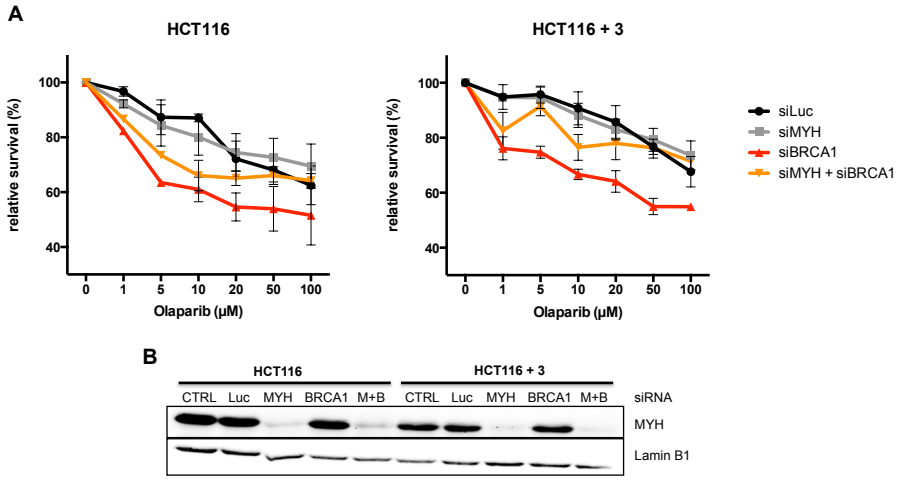

Fig.2: MYH co-depletion partially rescues Olaparib sensitivity of various BRCA1-depleted and BRCA1-deficient cell lines. A) Cell Titer Blue survival assays of MMR-deficient HCT116 colon cancer cells and MMR-proficient HCT116 cells that were complemented with part of chromosome 3 (HCT116 + 3). Cells were transfected with siRNA targeting MYH, BRCA1 or a combination of both and treated with increasing doses of Olaparib for 72h. Survival of untreated cells was set to 100%. Data are represented as mean ± SD (n = 2) B) Western Blot analysis of MYH knock-down efficiency in HCT116 and HCT116 + 3 cells.

MYH-depletion rescues SSB and DSB accumulation in Olaparib exposed cells

The toxicity of PARP inhibitors is thought to arise from an accumulation of DNA breaks that cannot be repaired faithfully in cells lacking functional HR. We therefore monitored DNA strand break accumulation at single cell level using an alkaline comet assay. In agreement with our hypothesis, compared to BRCA1 depletion alone, we observed a marked reduction of DNA breaks upon MYH and BRCA1 co-depletion in A2780 cells treated with 10 µM Olaparib for 24 hours (Fig. 3 A&B). Next, we made use of the pulse field gel electrophoresis (PFGE) technique to specifically quantify DNA double strand breaks. Also here we found significantly reduced levels of DSBs in MYH and BRCA1 co-depleted samples upon exposure to Olaparib (Fig. 3 C&D). Notably, MYH-depletion by itself also seems to reduce the amount of SSBs and DSBs in genomic DNA compared to control cells (siLuc), although to a slightly lower degree than in BRCA1 co-depleted cells. Collectively, these results suggest that MYH-initiated repair of oxidative DNA damage creates transient SSBs, which likely require functional PARP activity for repair. As a result of PARP inhibition by Olaparib, these SSBs accumulate in the genome and eventually collapse into deleterious DSBs, which cannot be repaired faithfully in BRCA1-deficient cells.

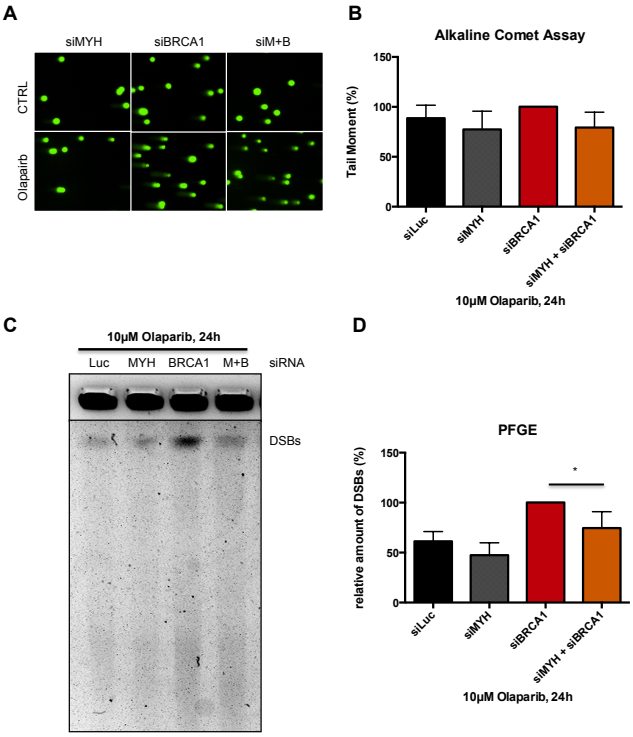

Fig.3: MYH depletion partially reduces DNA strand breaks upon Olaparib treatment of BRCA1-depleted A2780 cells. A&B) Representative images and quantification of three independent Alkaline Comet experiments in A2780 cells depleted of MYH, BRCA1 or a combination of both treated with 10 µM Olaparib for 24h. Relative Tail Moment (%) was normalized to siBRCA1 samples, which was set to 100%. C&D) Representative image and quantification of three independent Pulse Field Gel Electrophoresis (PFGE) experiments in A2780 cells upon Olaparib treatment (10 µM) for 24h. Relative amount of DSBs (%) was normalized to siBRCA1 samples, which was set to 100%. M+B: cells transfected with a combination of siRNA targeting MYH and BRCA1.

MYH-depletion leads to PARP inhibitor resistance in HR-deficient cells

Besides BRCA1 and BRCA2, also other factors involved in homologous recombination were shown to induce synthetic lethality together with PARP inhibitors. In order to test, whether the reduced sensitivity and genomic instability upon MYH depletion is due to a general defect in HR, we repeated the above-mentioned experiments in Rad51-depleted and MYH + Rad51 co-depleted A2780 cells and were able to recapitulate the results obtained previously using siRNA targeting BRCA1. Loss of MYH leads to Olaparib resistance, and reduces the accumulation of SSBs as well as DSBs in genomic DNA in a Rad51-deficient background (Fig. 4 A-F).

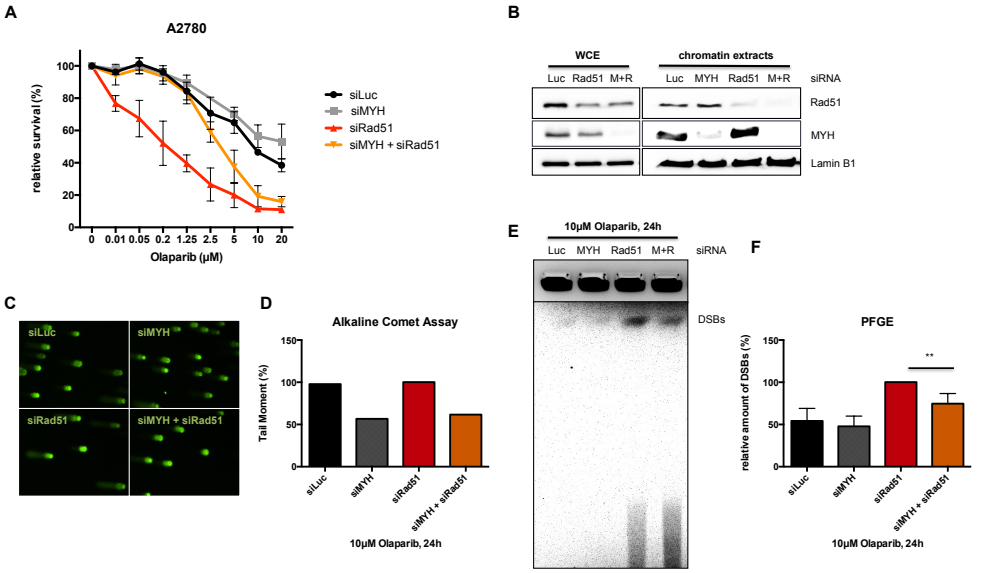

Fig.4: MYH co-depletion partially rescues Olaparib sensitivity of HR-deficient A2780 cells. A) Cell Titer Blue survival assays of A2780 cells depleted of MYH, Rad51 or a combination of both treated with increasing doses of Olaparib for 96h. B) Western Blot analysis of MYH and Rad51 knock-down efficiencies in whole cell extracts (WCE) and chromatin enriched samples. C&D) Representative images and quantification of one Alkaline Comet experiment in A2780 cells treated with 10 μM Olaparib for 24h. Relative Tail Moment (%) was normalized to siRad51 sample, which was set to 100%. E&F) Representative image and quantification of three independent Pulse Field Gel Electrophoresis (PFGE) experiments in A2780 cells upon Olaparib treatment (10 μM) for 24h. Smear in the lower part of the gel represent smaller DNA fragments, induced by genome fragmentation during apoptosis. Relative amount of DSBs (%) was normalized to siRad51 samples, which was set to 100%. M+R: cells transfected with a combination of siRNA targeting MYH and Rad51.

Loss of MYH does not lead to growth defects in Rad51-depleted A2780 cells

To rule out that proliferation defects are responsible for the above-mentioned MYH-induced rescue of Olaparib toxicity, we investigated growth patterns and cell cycle distribution of siRNA-depleted cells. Although Rad51 knock-down reduces overall growth rates in mock-treated A2780 cells, additional MYH depletion does not further reduce or accelerate the observed growth retardation (Fig. 5 A). In accordance with this phenotype, knock-down of Rad51, independently of MYH status, increases the population of cells in G2 phase of the cell cycle (Fig. 5 B&C). Furthermore, MYH knock-down had no effect on growth rates or cell cycle distribution in Rad51-depleted cells that were exposed to the PARP inhibitor Olaparib (Fig. 5 D-F).

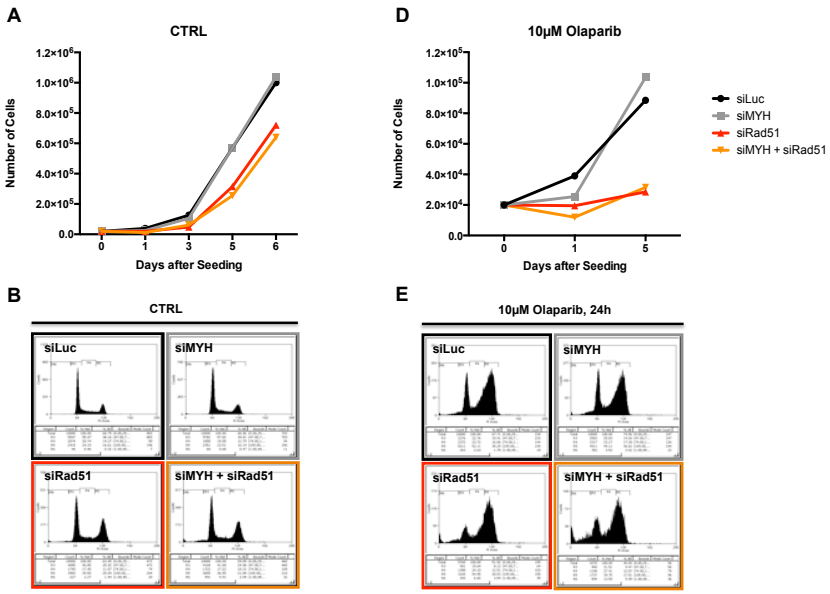

Fig.5: MYH depletion does not cause further growth retardation or acceleration in Rad51-depleted A2780 cells. A) After seeding 20000 cells on Day 0, the total number of control cells was counted over the course of six days. B&C) Representative images of cell cycle profiles after Propidium Iodide staining and quantification of cells in the different cell cycle stages, respectively. D-F) Growth curve, FACS profiles and quantification of cells in different cell cycle stages upon Olaparib treatment at 10 µM for 24 hours to five days.

PARP1 trapping on endogenous DNA lesions mediates Olaparib toxicity

Trapping of PARP1 and PARP2 on DNA after lesion recognition was proposed to be the major mechanism mediating toxicity of several different clinical PARP inhibitors (39). According to this hypothesis, inhibition of PARP by a certain class of small molecule inhibitors prevents automodification, which is characterized by synthesis of highly negatively charged poly-ADP-ribose (PAR) polymers on itself and other acceptor proteins upon DNA damage binding. These PARP poisons thus interfere with the repulsion of the protein from DNA and it was shown that trapped PARP-DNA complexes are more cytotoxic than the original SSBs (39). Our results confirm this hypothesis. Compared to siRNA-mediated knock-down of PARP1 alone, loss of BRCA1 together with PARP1 yielded about 80% survival of untreated control cells (Fig. 6 A). Additionally, compared to BRCA1-depletion, simultaneous knock-down of BRCA1 and PARP1 leads to reduced Olaparib sensitivity (Fig. 6 B), suggesting that the absence of PARP1 confers Olaparib resistance because no PARP-DNA complexes will be formed. Knock-down of MYH in this background did not result in any additional effects on cell viability in control or Olaparib treated cells (Fig. 6 A&D). Although we cannot strictly rule out a contribution of PARP2 or PARP3, which are also inhibited by Olaparib, it seems that trapping of the protein on DNA at least partially contributes to its phenotype in our system.

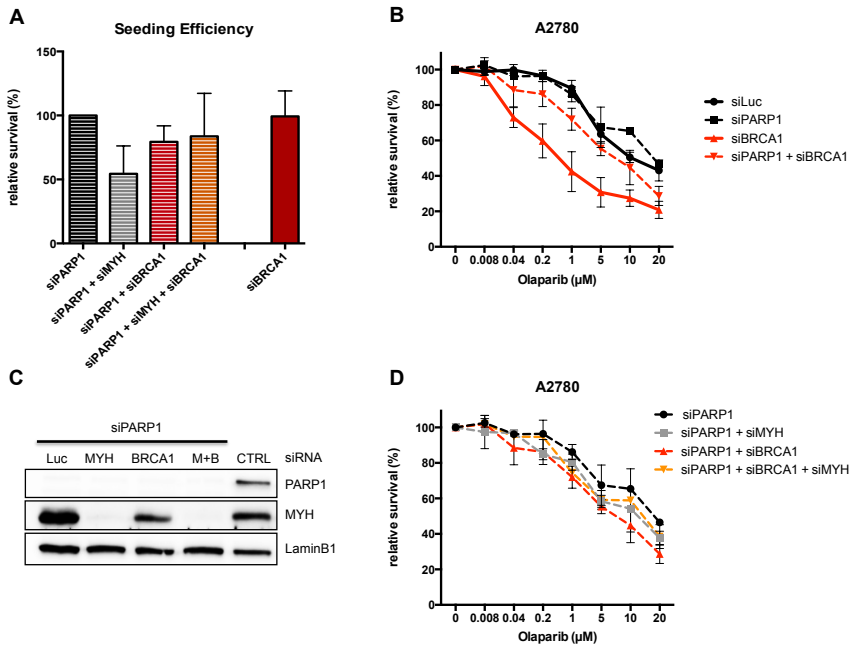

Fig.6: PARP1 depletion reduces Olaparib toxicity in BRCA1-depleted A2780 cells. A) Seeding efficiencies of A2780 cells depleted of PARP1 alone or in combination with MYH and/or BRCA1 as measured in untreated populations of two independent Cell Titer Blue survival experiments. The survival of siPARP1-transfected cells was set to 100%. Error bars represent SD (n = 2). B) Cell Titer Blue survival assay shows reduced Olaparib toxicity upon PARP1 and BRCA1 co-depletion. The survival of untreated cells was set to 100%. Error bars represent SD (n = 2). C) Western Blot analysis of PARP1 and MYH knock-down efficiencies. D) Cell Titer Blue survival assay of PARP1-depleted A2780 cells treated with increasing concentrations of Olaparib for 72h. The survival of untreated cells was set to 100%. Error bars represent SD (n = 3).

RNaseH2-mediated excision of genomic rNTPs does not contribute to PARP inhibitor sensitivity

Ribonucleotides (rNTPs) are frequently misincorporated into the DNA by replicative polymerases under physiological conditions and are likely the most abundant form of DNA damage in the genome (40,41). They are about 100000-fold more susceptible to spontaneous hydrolysis than deoxynucleotides due to the additional reactive hydroxyl group at the 2' position of the ribose sugar (42), which is why processing of such lesions by ribonuclease (RNase) H2 is required to preserve genomic stability (43). We thus reasoned that RNaseH2-mediated removal of genomic rNTPs could be another mechanism contributing to PARP inhibitor toxicity in HR-deficient cells. In order to test this, we knocked-down RNaseH2 together with BRCA1, but found that these cells are as sensitive to PARP inhibitor treatment as BRCA1-depleted A2780 cells (Fig. 7 A). Co-depletion of RNaseH2 and BRCA1 also resulted in even further increased DSB accumulation upon exposure to Olaparib (Fig. 7 C), suggesting that spontaneous hydrolysis of rNTPs leads to enhanced genomic instability in the absence of RNaseH2. These results also imply that, unlike MYH-induced BER of G^{O}:A base-pairs, processing of rNTPs by RNaseH2 does not generate transient repair intermediates that require PARP activity. This highlights that processing only of a specific subset of endogenous DNA lesions induces PARP inhibitor sensitivity in HR-deficient cells, which likely depends on the molecular repair mechanism.

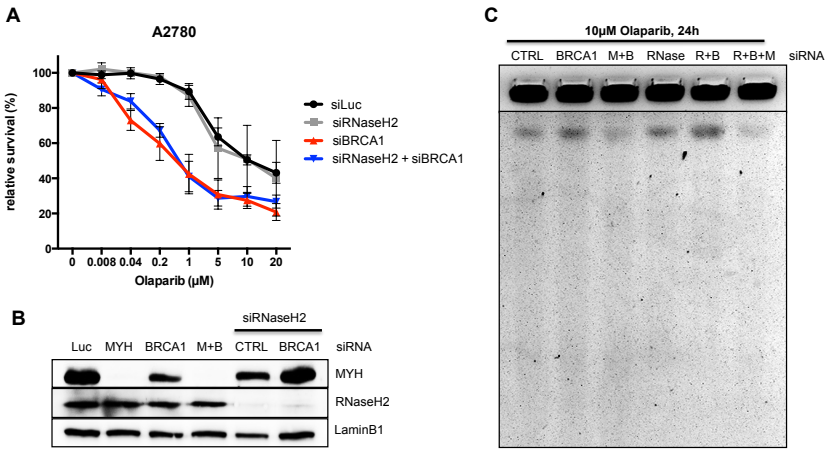

Fig.7: RNaseH2 co-depletion does not rescue Olaparib sensitivity of BRCA1-depleted A2780 cells. A) Cell Titer Blue survival assays of A2780 cells transfected with siRNA targeting RNaseH2, BRCA1 or a combination of both treated with increasing doses of Olaparib for 72h. Survival of control cells was set to 100%. Data are represented as mean ± SD (n = 2) B) Western Blot analysis of MYH knock-down efficiency. C) Cell Titer Blue survival assays A2780 cells transfected with siRNA targeting RNaseH2, BRCA1 and MYH or a combination of those treated with increasing doses of Olaparib for 72h. D) Representative image of PFGE analysis to detect DSBs in response to treatment with 10 μM Olaparib for 24h.

Discussion

The use of PARP inhibitors to specifically target BRCA1 or BRCA2 deficient ovarian and breast cancer cells is currently tested in clinical trials. Olaparib is the first PARP inhibitor that was approved for clinical use. It is authorized as fourth-line treatment for advanced BRCA-mutated ovarian cancer. However, (acquired) resistance to these small molecule PARP inhibitors has already been proposed to be a major obstacle, affecting their efficacy and clinical outcome. For instance, increased drug efflux by overexpression of multi-drug resistance (MDR) transporters or reduced expression of PARP1 may diminish the efficacy of Olaparib and other PARP inhibitors (44). Furthermore, secondary mutations that restore functional BRCA1 or BRCA2 proteins, as well as inhibition of the NHEJ pathway due to mutations or loss of 53BP1 or Rev7 (also known as MAD2L2) can reactivate HR, rendering cells insensitive to PARP inhibitor treatment (3,44-48). We hypothesized that loss of other factors, which normally contribute to PARP inhibitor sensitivity in HR-deficient cells, could lead to drug resistance. Likely candidates appeared DNA repair proteins, which are involved in processing of endogenous DNA damage. We focused on oxidative lesions due to the increased levels of ROS in cancer cells and found that loss of the BER glycosylase MYH, which is crucial for the repair of potentially mutagenic G^{o}:A base-pairs, partially inhibits Olaparib toxicity in HR-deficient cells. Our results indicate that MYH-depletion reduces the number of transient SSBs, which are normally generated by the combined actions of MYH and APE1 upon excision of misincorporated adenines opposite G^{o} and subsequent cleavage of the sugar-phosphate backbone of the DNA. PARP1 may recognize these transient repair intermediates and facilitate fast and efficient repair. Inhibition of PARP will consequently interfere with the repair process and lead to their collapse into one-ended DSBs or induce the formation of DNA-PARP complexes, which are highly cytotoxic. Our results highlight the importance of oxidative DNA damage repair initiation for the efficacy of PARP inhibitors in HR-deficient cells. These findings could

have important implications for personalized medicine, considering that the application of PARP inhibitors in hypoxic tumor regions might be ineffective due to reduced oxidative pressure and therefore reduced number of oxidative DNA lesions that may contribute to PARP activation. Furthermore, one may envision that low expression or mutation of MYH in tumors, such as MYH-associated polyposis (MAP), could lead to PARP inhibitor resistance and therefore serve as a suitable prognostic biomarker in the clinic. However, detailed bioinformatic analysis of patient data will be required to confirm this hypothesis in the future.
Besides oxidative DNA damage, also other types of endogenous DNA damage could potentially contribute to PARP inhibitor toxicity in HR-deficient cells. Specifically, we investigated ribonucleotide processing by RNaseH2 due to the vast abundance of rNTPs in genomic DNA. However, we found that knock-down of this enzyme had no protective effect towards survival and DSB accumulation upon Olaparib treatment in BRCA1-depleted cells. This emphasizes that different endogenous DNA lesions are repaired by distinct mechanisms, which likely do not all trigger PARP activation and can thus be neglected regarding the efficacy of PARP inhibitor therapy. However, considering the variety of different endogenous lesions, the question raises, whether also other repair factors, besides MYH, could contribute to PARP inhibitor sensitivity. This will be part of our future investigations.

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RESULTS II

The role of ATAD5 in genome stability

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Abstract

Elg1 (Enhanced level of genome instability) has been described as a suppressor of genome instability in yeast, however, very little is known about its human homolog ATAD5. Both are constituents of alternative replication factor complexes (aRFC), in which they replace the large subunit RFC1 to associate with the four small subunits RFC2-5 [1, 2]. Due to accumulation of PCNA and ubiquitylated PCNA on DNA upon depletion of Elg1 and ATAD5, both aRFC complexes, Elg1/RFC2-5 and ATAD5/RFC2-5, have been proposed to act as PCNA unloaders in yeast and human cells, respectively. As PCNA plays a central role in orchestrating DNA replication and repair events [3], its controlled removal from DNA may be crucial to maintain genome integrity. Interestingly, Elg1- as well as ATAD5-deficient cells display hypersensitivity to certain DNA damaging agents [4, 5]. These results demonstrate that Elg1 and ATAD5 are involved in DNA repair and may prevent mutagenesis in response to DNA damage.

We set out to elucidate the role of human ATAD5 in genome maintenance, specifically focusing on its basic biological function in DNA metabolism. In order to do so, we investigated replication and repair in ATAD5-depleted human cells under native conditions and upon exposure to DNA damaging agents. Similarly to yeast [4, 5], we found hypersensitivity of ATAD5-deficient cells towards DNA alkylating agents, such as MNNG and MMS. Furthermore, ATAD5-depleted cells were sensitive to treatment with the DNA crosslinking agent MMC and the PARP1 inhibitor Olaparib. Notably, the enhanced sensitivity to DNA damaging agents correlated with an accumulation of DNA double-strand breaks or apoptosis, demonstrating that ATAD5 is indeed required to maintain genome integrity. In addition, we confirm an accumulation of PCNA and $PCNA^{ub}$ on chromatin upon ATAD5 depletion; however, this did not lead to any defect in replication, suggesting that ATAD5 is not involved in replication *per se*. Collectively, our data confirm a role for the human ATAD5 in genome maintenance upon exposure to DNA damaging agents. However, thus far we were not able to prove whether its molecular function is to unload PCNA or $PCNA^{ub}$ from DNA to protect the genome.

Introduction

Our genome is constantly exposed to a variety of endogenous and exogenous DNA damaging agents that may cause mutations if not corrected before replication. In order to protect the genetic material from mutagenesis, a network of repair pathways has evolved. A novel factor involved in maintaining genome stability was identified in yeast genetic screens and named Enhanced level of genome instability (Elg1) [2, 4, 6]. Yeast strains lacking Elg1 (*Δelg1*) are hypersensitive to certain DNA damaging agents and exhibit increased levels of (gross) chromosomal rearrangements, as well as recombination events [4, 7]. Additionally, Elg1 was shown to be involved in telomere maintenance, sister chromatid cohesion and DNA replication [6, 8, 9]. However, its precise function in DNA repair, especially its role in maintaining genome stability in human cells, still remains elusive.

Elg1, like its human homolog ATAD5, is part of an alternative replication factor C (RFC) complex that is composed of five subunits Elg1/RFC2-5 or ATAD5/RFC2-5, respectively [1, 2, 4]. Elg1 or ATAD5 replace the large subunit RFC1 of the canonical RFC (RFC1/RFC2-5) complex that loads proliferating cell nuclear antigen (PCNA) onto DNA

at primer-template junctions to facilitate replication [10]. RFC1 can also be replaced by two other proteins, namely Rad17 (RAD24 in human cells) or Ctf18. Rad17/RFC2-5 and Ctf18/RFC2-5 are involved in Rad9-Rad1-Hus1 (9-1-1) complex loading at sites of DNA damage [11] and sister chromatid cohesion [12], respectively. The Elg1/RFC2-5 and ATAD5/RFC2-5 complexes seem to be required for the unloading of PCNA from chromatin in yeast as well as in human cells [13, 14]. Direct biochemical evidence for such a function is still missing, but in yeast this hypothesis was supported by the fact that purified Elg1/RFC2-5 is able to unload PCNA from chromatin extracted from an Elg1-deficient yeast strain [13]. Recent evidence also suggests that Elg1/RFC2-5 is required for global PCNA unloading after Okazaki fragment ligation throughout the genome [15], rather than solely from specific unloading at sites of DNA damage or cohesion. In human cells, indirect evidence was provided by experiments showing that PCNA accumulates on DNA in the absence of ATAD5 [14, 16]. Furthermore, the lifespan of active replication factories on DNA was extended even into G2 phase of the cell cycle [14]. ATAD5 was proposed to maintain low levels of ubiquitinylated PCNA on chromatin by recruiting ubiquitin-specific protease 1 (USP1) and its associated factor (UAF1) to ubiquitinylated PCNA [16]. Interestingly, ubiquitylation of PCNA is required for translesion synthesis (TLS) over certain DNA lesions [17]. Removal of $PCNA^{ub}$ from chromatin by ATAD5 may therefore inhibit error prone TLS and promote more accurate repair pathways.
Despite the advances in the field, the exact molecular role of ATAD5 in DNA metabolism is still unclear. It also remains enigmatic how removal of PCNA or $PCNA^{ub}$ contributes to maintain genome stability. The aim of this study was thus to assess the role(s) of human ATAD5 in DNA repair and replication, especially with respect to its potential role as a PCNA unloader. Our results confirm that ATAD5-deficient cells are hypersensitive to a subset of DNA damaging agents and accumulate more strand breaks upon exposure to those drugs. Despite the accumulation of PCNA and $PCNA^{ub}$ on chromatin, replication is not affected by ATAD5 depletion, irrespective of whether the genome is intact or damaged. Further experiments are required to clarify whether unloading of PCNA or $PCNA^{ub}$ is the main function of ATAD5 and how exactly unloading of PCNA promotes genome stability.

Material and Methods

Cell culture and shRNA-mediated downregulation of ATAD5
A2780 cells were stably transfected with two different expression plasmids containing shRNAs targeting ATAD5 (1-6, targeting a sequence in exon 2: 5'-CUGACGAUGUACAAGAUAAUA-3' and 15-3, targeting a sequence in the 3'UTR: 5'-GUAUAUUUCUCGAUGUACA-3'). Cells were grown in DMEM (Gibco), complemented with 5% Tet-approved fetal calf serum (FCS, Sigma), penicillin (100 U/ml, Gibco) and streptomycin (100 µg/ml, Gibco), Blasticidin S (10 mg/ml, InvivoGen) and Puromycin (10 mg/ml) at 37°C in a 6% CO_2 humidified atmosphere. For induction of ATAD5 knock-down, cells were exposed to 100 ng/ml doxycycline (Clontech) for at least four days.
HEK293 TRex FlpIN cells were cultivated in DMEM, supplemented with 10% FCS (Gibco), Blasticidin S (10 mg/ml, InvivoGen) and Puromycin (10 mg/ml) at 37°C in a 6% CO_2 humidified atmosphere. Knock-down of endogenous PCNA or ATAD5 was achieved by addition of 100 ng/ml doxycycline (Clontech) to the cell culture medium for at least three days.

Western Blot
SDS lysates were prepared using 2x Laemmli Buffer (4% SDS, 20% glycerol, 120 mM Tris pH 6.8) and boiled for 5 min at 95°C before sonication (20 sec, 50 cycles, 70% amplitude; Bandelin Sonoplus GM70). Protein concentration was determined using the

Lowry assay: 3 µl sample were diluted in 97 µl H_2O and incubated for 10 min with 1 ml of a 50:1 mixture of Solution A (2% Na_2CO_3 in 0.1 N NaOH) and Solution B (0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium citrate). 100 µl of a Folin&Ciocalteau's phenol reagent (Sigma) and H_2O dilution (1:2) were added to the mixture and incubated for 1h at room temperature (RT). Absorbance was measured at 750 nm (Varian-Cary 50 Scan spectrophotometer) and protein concentration calculated according to a calibration curve using increasing concentrations of a BSA standard.
25-50 µg protein were supplemented with 5x Loading Buffer (0.25 M Tris pH 6.8, 50% glycerol, 8% SDS, 0.5 mM DTT, 0.1% bromphenol blue) and separated according to size on acylamide gels (6% for ATAD5) using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) in 10% SDS-buffer at 100 V. Proteins were transferred in Transfer Buffer (25 mM Tris, 192 mM glycine, 10% methanol) overnight at 4°C to a Hybond-P Polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia Biotech) that was previously activated in 100% methanol. Membranes were blocked in 5% non-fat dry milk in 1x TBS-T (20 mM Tris-HCl pH 7.4, 150 nM NaCl and 0.1% Tween-20) for at least 30 min at roomtemperature (RT) before incubation with primary antibodies overnight at 4°C in 5% milk, washing 3x with TBS-T and subsequent incubation with secondary antibodies (horseradish peroxidase (HP)-conjugated sheep anti-mouse or donkey anti-rabbit IgG, GE Healthcare) for 1h at RT. After three washes in TBS-T, membranes were incubated for 1 min with WesternBright™ Chemiluminescent Detection Reagent (Advansta) (800 µl H_2O mixed with 200 µl of both reagents) and analyzed with a Fusion Solo (Vilber Lourmat).

Clonogenic survival assays
Four days after exposure to doxycycline (100 ng/ml), cells were seeded into 6-well plates at a concentration of 300 cells per well, prior to treatment with DNA damaging agents the following morning. For MNNG, cells were pre-treated with O^6-benzylguanine (O^6-BG) for 1 h at 10 µM, before addition of MNNG for the remaining duration of the experiment. Cells were exposed to mitomycin C (MMC) or hydroxyurea (HU) for 48 h or 24 h, respectively, before washing out the drug 1x with PBS and subsequent addition of normal cell culture medium for the remaining duration of the experiment. Cells were continuously treated with Olaparib, camptothecin (CPT), TMPyP4 (Santa Cruz) and S2T1-6OTD (kind gift of K. Nagasawa).
Colonies were stained 10 days after exposure to the drug with 0.5% Crystal Violet (MERCK) in 20% ethanol and depicted as relative survival fraction (%) compared to control cells (set to 100% survival) using GraphPad Prism 6.

Pulse Field Gel Electrophoresis Assay
Cells were cultivated with or without 100 ng/ml doxycycline for at least four days before seeding in 10 cm dishes to reach 60% confluence on the day of the treatment. For MNNG treatment, cells were pre-exposed to 10 µM O^6-BG and then treated with the indicated concentrations of MNNG for 24 h, 48 h or 96 h. Cells were treated with 0.5 µM MMC for 96 h, 2 µM Olaparib for 24 h or 50 nM camptothecin (CPT) for 24 h. At the indicated time points, the supernatant was collected and cells were harvested by trypsinization. Cells were pelleted, washed with PBS and counted using the Countess Automated Cell Counter (Invitrogen) and 0.4% Trypan Blue stain (Invitrogen). For preparation of one plug, 250'000 cells were transferred to a new tube, spun down and resuspended in 50 µl PBS. Subsequently, cells were embedded in 1.5% low melting agarose (Sea Plaque GTG Agarose, Lonza) at 50°C, pipetted into CHEF plug molds (BioRad) and subsequently incubated in Lysis Buffer (100 mM EDTA pH 8.0, 0.2% Sodium Deoxycholate, 1% Sodium Lauryl Sarcosine, 20 mg/ml Proteinase K, Applichem) for 36-72 h at 37°C. After washing 3x for 30min with 5 ml washing buffer (20 mM Tris-HCl, 50 mM EDTA pH 8.0), plugs were loaded in wells of a 0.9% Pulse Field Certified Agarose gel (BioRad) in 0.5% TBE buffer. Electrophoresis was carried out in a CHEF-DR

III Pulse Field Gel Electrophoresis System (BioRad) at 14°C: block I: switch time 30-18 s, run time 9 h, angle 120°, voltage gradient 5.5 V/cm; block II: switch time 18-9 s, run time 6 h, angle 117°, voltage gradient 4.5 V/cm; block III: switch time 9-5 s, run time 6 h, angle 112°, voltage gradient 4 V/cm. Gels were stained in 0.5% TBE buffer containing 0.5 µg/ml Ethidium bromide (Sigma) and analyzed on an Alpha Innotech imaging system.

Alkaline Comet Assay
Alkaline Comet Assays were performed as described in CometAssay® (Trevigen). Briefly, cells were pre-exposed to doxycycline for at least four days prior to treatment with 3 mM MMC for 1 h. Samples termed 'recovery' were subsequently washed with PBS and cultured for 4 more hours in normal cell culture medium before trypsinization. Cells (at a concentration of $1x10^5$/ml) were embedded in molten LMAgarose (Trevigen) at 37°C (ratio 1:10 (v/v)) and 50 µl of this suspension were immediately pipetted onto CometSlides™. Slides were chilled for 10 min at 4°C before immersion in Lysis Solution (Trevigen) for 30-60 min at 4°C and subsequent irradiation with 5 Gy to enhance background levels of '% comet tail DNA'. DNA was unwound in Alkaline Unwinding Solution, pH>13 (300 mM NaOH, 1 mM EDTA) for 20 min at 4°C in the dark and electrophoresis was performed at 21 V for 30 min in a CometAssay® ES unit in 850 ml chilled Alkaline Electrophoresis Solution, pH>13 (300 mM NaOH, 1 mM EDTA). Slides were washed twice in dH_2O for 5 min each, then once in 70% ethanol, before drying completely at 37°C and staining with SYBR® Green for 30 min at RT. Again, slides were rinsed quickly in dH_2O and dried completely prior to analysis on a Olympus IX81 fluorescence microscope.

RNA interference
Cells were transfected at a densitiy of 30-50% using RNAiMAX (Invitrogen) according to manufacturer's instructions. For 6-well plates, 30 pmol siRNA (siATAD5 15-3: 5'-GUAUAUUUCUCGAUGUACA-3') were diluted in 250 µl OptiMEM (Invitrogen) and mixed well with 250 µl OptiMEM supplemented with 4 µl RNAiMAX transfection reagent. After incubation at RT for 15 min, the mixture was added drop-by-drop to the cells and incubated for at least 72 h.

Immunofluorescence
Cells grown on cover slides were washed once with PBS and soluble proteins were removed by incubation with Preextraction Buffer (25 mM Hepes pH 7.4, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM Sucrose and 0.5% Triton-X-100) for 5 min at 4°C (chromatin-bound protein fraction is retained). Subsequently, cells were washed twice with ice-cold PBS and fixed in 3.7% formaldehyde (Sigma) for 10 min at RT. Slides were then washed 3x with PBS before blocking in 5% BSA in PBS for 30 min at RT and subsequent incubation with primary antibodies in blocking solution at 4°C overnight (γH2AX: 1:500, rabbit polyclonal, Cell Signaling; PCNA sc-56: 1:100, mouse monoclonal, Santa Cruz). Cells were washed again 3x with PBS, incubated with appropriate secondary antibodies in blocking solution (FITC-conjugated anti-rabbit antibody, 1:500, Sigma and TR-conjugated anti-mouse antibody, 1:500, Abcam) for 1 h at RT, washed twice with PBS, once with ddH_2O and mounted on microscope slides (Thermo Scientific) with Vectashield mounting medium containing DAPI (Vector Laboratories). After sealing, images were taken at a 60x magnification on an Olympus IX81 fluorescence microscope.

FACS Analysis
Asynchronous cell population treated with or without doxycycline for four days were grown in 6-well plates to reach 70% confluency before harvesting by trypsinization. Cells were pelleted, washed once with ice-cold PBS and fixed with 80% ethanol prior to

staining with 100 µg/ml Propidium Iodide (Sigma) in PBS supplemented with 20 µg/ml RNase A (Sigma) for 30min at RT. The Cyan ADP9 Flow Cytometer (Beckman Coulter) was used and DNA content in different cell cycle phases quantified using Summit v4.3.01 software.
For cell synchronization experiments, cells were arrested in G1 phase by double thymidine block (DTB). Briefly, 2 mM thymidine (SynGen Inc.) was added to cells grown at a confluence of 60%. 16h later, thymidine was washed out with PBS (3x), cells were released into medium without thymidine for 8 h and then thymidine (at a final concentration of 2 mM) was added again for another 16 h. One hour after the second release, 10 µM O^6-benzylguanine (O^6-BG) was added, cells were washed 3x with PBS and released into normal medium supplemented with 1 µM MNNG and 10 µM O^6-BG. At the indicated time points cells were harvested and stained for FACS analysis as described above.

Chromatin extraction
Cells were treated as described above in the FACS analysis cell synchronization section, prior to chromatin extraction. Briefly, 1.5 Million cells per 10 cm dish were washed once with ice-cold PBS before incubation with Preextraction Buffer (25 mM Hepes pH 7.4, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM Sucrose, 0.5% Triton-X-100 and 1x complete, EDTA-free Roche protease inhibitor cocktail) for 12 min at 4°C. Cells were washed again 2x with PBS and afterwards scraped in 100 µl Laemmli Buffer, boiled for 5min at 95°C and sonicated for 25 sec (50 cycles, 70% amplitude). Protein concentration was assessed by Lowry assay and Western Blot performed as previously described.

DNA Fibre Assay
Cells were grown in 6-well plates to 70% confluence before treatment or labeling with nucleoside analogs as indicated. 4 µl CldU (5 mg/ml, Sigma) were added to cells grown in 2.5 ml medium and incubated for 30 min if not otherwise stated. CldU was washed out with equilibrated medium and replaced by medium containing 1 mM IdU (Sigma) for another 30 min, or as indicated. Subsequently, cells were washed 3x with ice-cold PBS, scraped and 2 µl of a cell suspension containing $1*10^6$ cells/ml were pipetted onto a glass slide (Super Frost Plus, Menzel-Glaser). Slides were dried for approximately 4 min until 'sticky', before swirling cells in 7 µl Lysis Buffer (200 mM Tris-HCl pH 7.4, 50 mM EDTA, 0.5% SDS) and incubation for another minute. Subsequently, slides were tilted slightly to let drops run down slowly, air-dried and fixed in methanol/acetic acid (3:1) for 10 min at RT. Slides were again air-dried and stored at 4°C. Prior to staining, slides were re-hydrated with PBS twice for 3 min, DNA was denatured in 2.5 M HCl for 1 h, washed 5x in PBS for 3 min each and immersed in blocking solution (2% BSA, 0.1% Tween 20, 1x PBS; 0.22 µm filtered) for 40 min. After incubation with primary antibodies diluted in blocking solution (rat anti-BrdU/CldU, 1:500, Abcam; mouse anti-BrdU/IdU, 1:100, Becton Dickinson) for 2.5 h at RT, slides were washed 5x 3 min in PBS supplemented with 0.2% Tween-20 (PBS-T) and washed once briefly in blocking solution. Subsequently, slides were incubated with secondary antibodies (anti-rat Cy3, 1:300, Jackson Immuno Research; anti-mouse Alexa 488, 1:300, Molecular Probes) for 1 h at RT, washed 5x with PBS-T for 3 min each and mounted with Vectashield without DAPI (Vector Laboratories). DNA fibre tracks were analyzed on an Olympus IX81 fluorescence microscope and track length determined using ImageJ software. At least 100 fibres were analyzed per experiment and graphs generated using GraphPad Prism 6 of at least two independent biological replicates.

Results

ATAD5-deficient cells are hypersensitive to DNA damaging agents

Elg1 was initially identified as a suppressor of genomic instability in yeast genetic screens [4, 5]. In order to test whether the mammalian homolog, ATAD5, has a similar protective function in human cells, we performed clonogenic survival experiments using different DNA damaging compounds with various modes of action. These assays were performed in a doxycycline-inducible A2780 ovarian carcinoma cell lines that stably harbor two different shRNAs targeting ATAD5. Cells were cultured in the absence or presence of doxycycline (Dox) for at least four days to downregulate the protein prior to treatment with the indicated drugs (Fig. 1 A). We detected sensitivity of ATAD5-depleted human cells to the methylating DNA agent MNNG only when cells have been pre-exposed to the O^6-Methylguanine-DNA-Methyltransferase (MGMT) inhibitor O^6-benzylguanine (O^6-BG) (Fig. 1 B). MGMT is a DNA repair enzyme that catalyzes the transfer of methyl groups from DNA bases onto itself and thereby inactivates itself. The fact that ATAD5-deficient cells are only sensitive to MNNG treatment when MGMT is inhibited led us to the assumption that ATAD5 may be linked to mismatch repair (MMR), which acts on certain methylated DNA bases, especially when they persist in the genome and give rise to mispairs during replication.

Furthermore, we observed that A2780 cells depleted of ATAD5 are hypersensitive to mitomycin C (MMC), an interstrand crosslinking reagent, and Olaparib, a PARP inhibitor (Fig. 1 C&D). These results hint towards a role of ATAD5 in homologous recombination (HR), since HR is ultimately required for accurate repair of interstrand crosslinks. Olaparib inhibits the single-strand break (SSB) repair enzyme PARP1 and thus induces persistent DNA SSBs, which may, upon encounters with the replication machinery, collapse into double-strand breaks (DSBs) that also require HR for accurate repair. However, double-strand break reporter assays using ATAD5-depleted cells were inconclusive and varied on the siRNA sequence (data not shown). We therefore cannot conclude that ATAD5 has a direct role in HR.

In contrast, knock-down of ATAD5 had no effect on survival of cells treated with replication inhibitors camptothecin (CPT) and hydroxyurea (HU), or the G4-quadruplex stabilizers TMPyP4 and S2T (Fig. 1 E-H), or hydrogen peroxide (H_2O_2, data not shown), which induces oxidative DNA damage and strand breaks. Collectively, these results suggest that ATAD5 is important for the maintenance of genome stability upon induction of different DNA lesions, however, it may not be directly involved in replication or telomere maintenance.

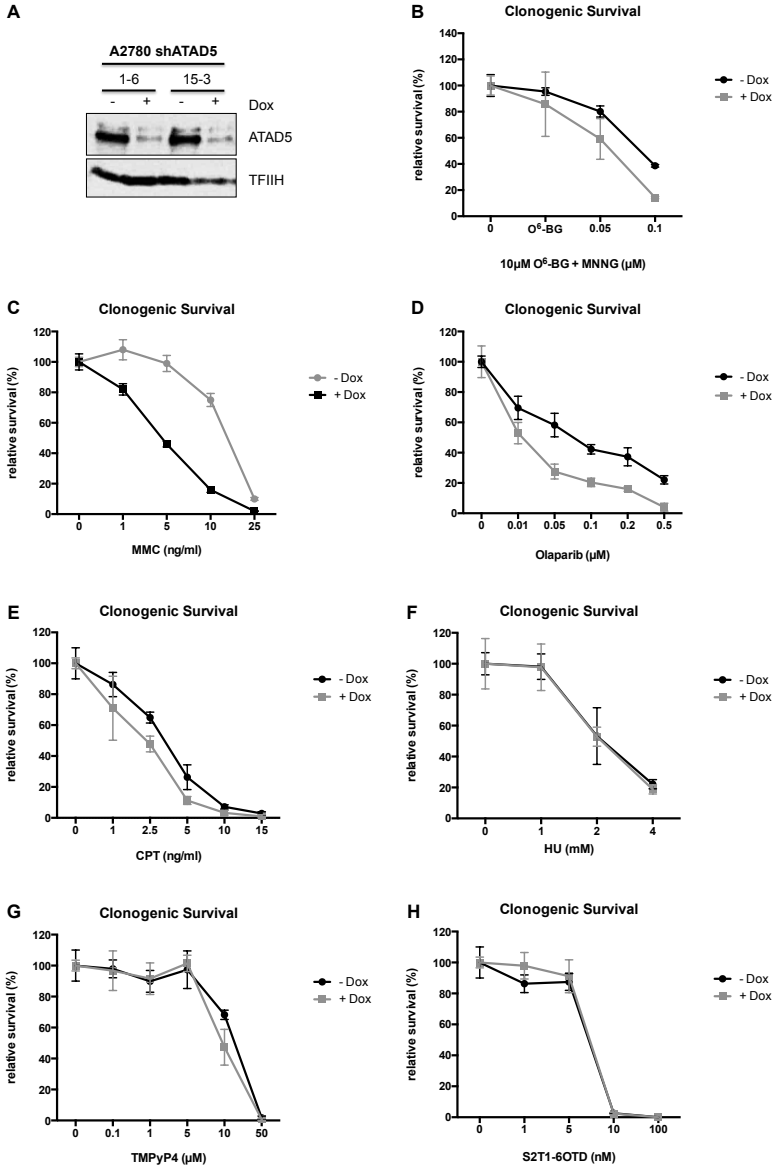

Fig.1 A) Representative Western Blot of doxycycline-inducible ATAD5 knock-down in stable clones of A2780 cells harboring either shRNAs 1-6 and 15-3. B) Clonogenic survival experiments using A2780 shATAD5 (15-3) cells pre-treated with 10 μM O^6-BG for 1h before addition of MNNG at the indicated concentrations for the remaining time of the experiment (10 days). C) Clonogenic survival experiment using A2780 shATAD5 (1-6) cells treated with the indicated concentrations of mitomycin C (MMC) for 48h. D) Clonogenic survival experiment using A2780 shATAD5 (1-6) cells continuously treated with the indicated concentrations of Olaparib. E) Clonogenic survival experiment using A2780 shATAD5 (1-6) cells continuosly treated with the indicated concentrations of camptothecin (CPT). F) Clonogenic survival experiment using A2780 shATAD5 (1-6) cells treated with the indicated concentrations of hydroxyurea (HU) for 24h. G) Clonogenic survival experiment using A2780 shATAD5 (1-6) cells continuosly treated with the indicated concentrations of TMPyP4. H) Clonogenic survival experiment using A2780 shATAD5 (1-6) cells continuously treated with the indicated concentrations of S2T1-6OTD. Depicted are representative results obtained from at least two independent experiments performed in triplicates.

DNA damaging agents cause slightly increased accumulation of DNA strand breaks in ATAD5-depleted cells

Due to the enhanced sensitivity of ATAD5-deficient cells to MNNG, MMC and Olaparib, we asked whether these agents would cause an accumulation of DNA double-strand breaks (DSBs) in these cells. In order to test this, we performed Pulse Field Gel Electrophoresis (PFGE) assays that allow separation of very large DNA fragments, such as broken chromosomes, from intact genomic DNA in agarose gels. Indeed, we observed that treatment with MNNG, MMC and Olaparib either increased the accumulation of DSBs (indicated by a high molecular weight band) or the fraction of apoptotic cells (indicated by the smear in the lower part of the gel, representing smaller DNA fragments generated during apoptosis) in ATAD5-depleted A2780 cells, as compared to their ATAD5-proficient counterparts (Fig. 2 A-C). These experiments therefore reflect the previously observed hypersensitivity to DNA damaging agents, which is likely a consequence of the enhanced genomic instability in the absence of ATAD5. ATAD5 status of A2780 cells had no significant impact on the accumulation of DSBs or apoptosis upon exposure to camptothecin (Fig. 2 D).

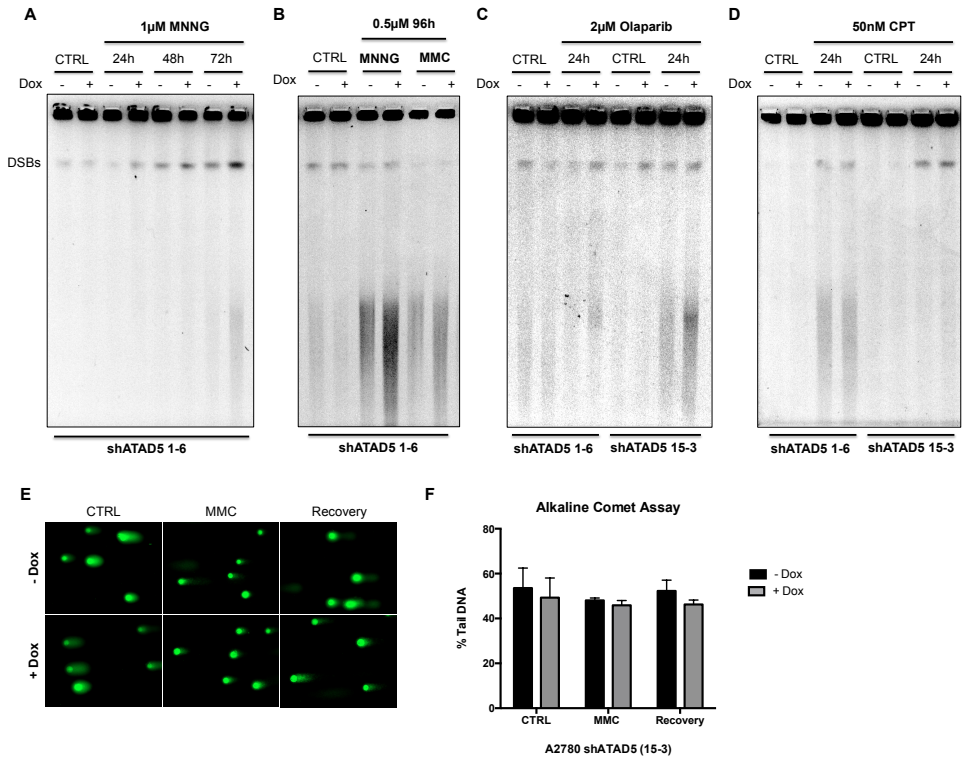

Fig.2 A) Representative image of a Pulse Field Gel Electrophoresis (PFGE) assay of A2780 shATAD5 (1-6) cells pre-treated with 10 µM O^6-BG before addition of 1 µM MNNG for 24h, 48h or 72h. B) PFGE of A2780 shATAD5 (1-6) cells using either 10 µM O^6-BG and 0.5 µM MNNG or 0.5 µM MMC for 96h. C) PFGE of A2780 shATAD5 (1-6 and 15-3) cells treated with 2 µM Olaparib for 24h. D) PFGE of A2780 shATAD5 (1-6 and 15-3) cells treated with 50 nM CPT for 24h. E) Representative images of comets in an Alkaline Comet Assay upon treatment with MMC for 1h and subsequent recovery for 4h. Cells were additionally irradiated using 5 Gy. F) Quantification of at least 60 comets per experiment of an Alkaline Comet Assay (error bars represent standard deviations (SD) calculated from the average '% Tail DNA' of two independent experiments).

Using the Alkaline Comet Assay we wanted to confirm on a single cell level that ATAD5-deficient cells have difficulties to repair DNA strand breaks induced by certain DNA damaging agents. The alkaline conditions allow to visualize not only DSBs but also SSBs in genomic DNA. MMC treatment and recovery therefrom indeed had a mild effect on the percentage of comet tail DNA in ATAD5-depleted cells (Fig. 2 E&F). MMC induces interstrand crosslinks (ICLs) and therefore reduces the mobility of DNA molecules in an electric field. Consequently, more DNA will remain immobile and stay in the nucleus represented by the comet head. Processing of ICLs during repair subsequently leads to the restoration of the background tail DNA amount. Our experiments show that the recovery from ICLs induced by MMC is abolished or delayed in ATAD5-deficient cells. However, further experiments are required in order to confirm this conclusion, especially analysis of longer recovery times after exposure to MMC and different doses of the drug. Additionally, it would be interesting to investigate the effect of other DNA damaging drugs in comet assays to confirm that DNA repair is indeed affected in ATAD5-depleted cells.

ATAD5 depletion causes retention of PCNA$^{(ub)}$ on chromatin and a delay in S-phase progression

It has already been proposed a few years ago that Elg1 and ATAD5 function as PCNA unloaders, most likely together with the small RFC2-5 subunits [2, 16]. However, biochemical assays that would provide experimental evidence for this hypothesis are still lacking, presumably due to missing co-factors or posttranslational modifications. More recently, a role for Elg1 and ATAD5 as PCNA unloaders has been strengthened by data showing that purified Elg1/RFC2-5 can indeed unload PCNA from chromatin extracted from a *Δelg1* yeast strain [13]. Furthermore, depletion of ATAD5 causes retention of PCNA on DNA and prolonged lifespan of replication factories into G2 phase of the cell cycle in human cells [14]. To test whether this is also true in our hands, we depleted ATAD5 in A2780 cells by doxycycline-inducible expression of an shRNA targeting the 3'UTR of ATAD5. Western Blots of whole cell extracts (Fig. 3 A) and immunofluorescence staining after pre-extraction (Fig. 3 B) confirmed that PCNA accumulates in A2780 cells upon ATAD5 knock-down. Interestingly, the amount of ubiquitylated PCNA (PCNA^{ub}) was also increased, as shown in HEK293 chromatin extracts transfected with siRNA targeting ATAD5 (Fig. 3 A).

Since PCNA accumulation on DNA could potentially create problems during replication, especially on the lagging strand, we asked whether the cell cycle of ATAD5-depleted cells is disturbed. FACS analysis with propidium iodide (PI) staining showed a slight increase in the S-phase population (Fig. 3 C), but not a profound block in any cell cycle phase, as also reported by others [13, 14]. However, this small S-phase retention did not result in any observable increase in DNA damage under control conditions, indicated by immunofluorescence staining of γH2AX, a marker for DNA strand breaks (Fig. 3 D). In order to gain a better understanding about how cells progress through the cell cycle, we released cells from a G1 double thymidine block (DTB) and simultaneously treated them with 1 µM MNNG in the presence of 10 µM O^6-benzylguanine. FACS profiles indicated that ATAD5-depleted cells progressed slightly slower through S-phase (apparent at the 6 h, 10 h and 12 h time points) compared to control cells under those conditions (Fig. 3 E). Notably, unmodified as well as ubiquitylated PCNA accumulated on chromatin if ATAD5 was missing, especially at early time points after release from the DTB (3 h and 6 h), when cells were progressing through S-phase (Fig. 3 F).

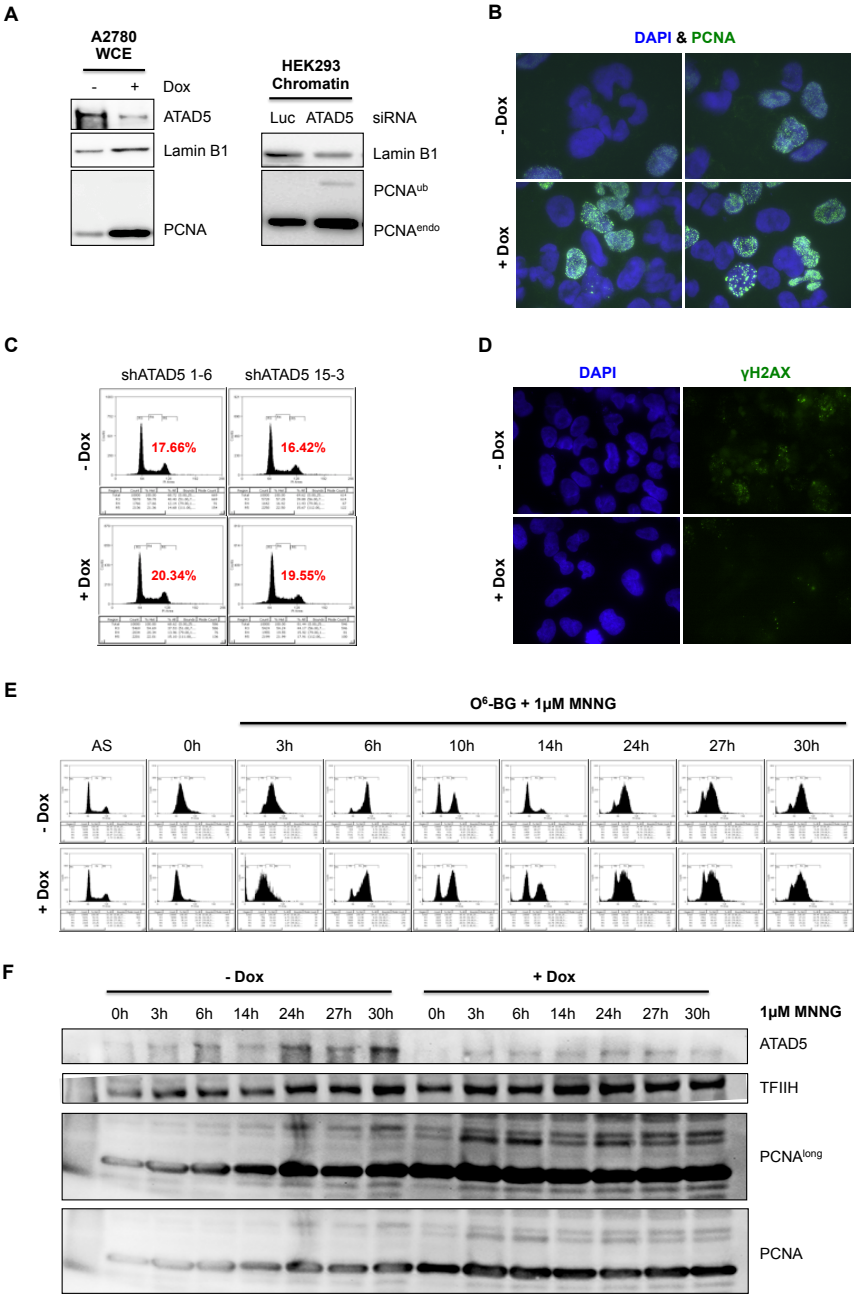

Fig.3 A) Western Blot of doxycycline-inducible ATAD5 knock-down in A2780 (15-3) cells and accumulation of PCNA in whole cell extracts (WCE). B) Immunofluorescence staining of pre-extracted A2780 shATAD5 (15-3) cells using DAPI and the anti-PCNA (sc-56) antibody. C) FACS cell cycle profiles of asynchronous A2780 shATAD5 (1-6 and 15-3) cells. The percentage of cells present in S-phase is indicated in red. D) Immunofluorescence imaging of A2780 shATAD5 (15-3) cells stained with DAPI and anti-γH2AX antibody. E) FACS cell cycle profiles of asynchronous (AS) population A2780 shATAD5 (15-3) cells and cells released from a double thymidine block (DTB). F) Western Blot analysis of chromatin extracted A2780 shATAD5 (15-3) cells that were pre-treated with 10 μM O^6-BG for 1 h before release from a DTB and simultaneous treatment with 1 μM MNNG for the indicated times.

To further investigate whether accumulation of unmodified PCNA or PCNA^{ub} causes genome instability, we generated a doxycycline-inducible HEK293 TRex FlpIN cell line in which we can simultaneously downregulate endogenous PCNA and overexpress either eGFP-tagged wild type PCNA (eGFP-PCNA_WT) or a PCNA mutant that cannot be ubiquitylated on lysine 164 (eGFP-PCNA_K164R). siRNA-mediated depletion of ATAD5 increased the retention of residual endogenous PCNA^{ub} on chromatin also in these cells (Fig. 4 A) and likewise increased the amount of exogenous wild type eGFP-PCNA^{ub} on DNA (Fig. 4 B). In contrast, the eGFP-PCNA_K164R mutant was as expected not ubiquitylated even in the absence of ATAD5 (Fig. 4 D).
Despite the incomplete knock-down of endogenous PCNA, these cell lines can serve as useful tools to investigate the importance of PCNA modification by ubiquitylation or SUMOylation on lysine 164 for the functional characterization of ATAD5 in maintaining genome stability. One may envision for instance that ATAD5 is merely required for the unloading of PCNA^{ub} to limit or prevent translesion synthesis (TLS) at stalled replication forks. On the other hand, ATAD5, as recently proposed for yeast Elg1, could be a general unloader that removes PCNA from DNA after Okazaki fragment ligation [15], possibly independently of its posttranslational modifications. Future experiments using these cells will show whether ATAD5 causes sensitivity to DNA damaging agents only in a setting where PCNA can become ubiquitylated, or if it is generally required to unload PCNA to prevent genome instability.

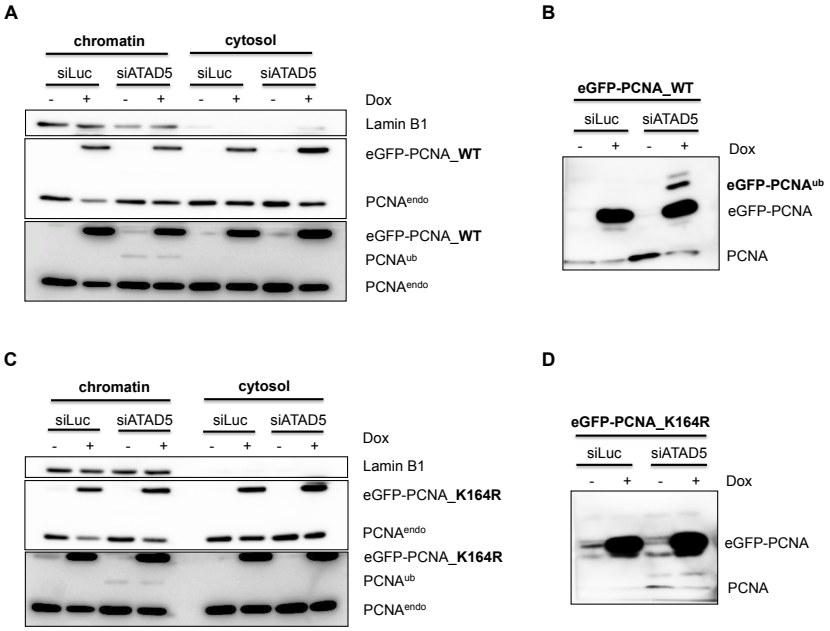

Fig.4 A) Western Blot analysis of chromatin and cytosolic fractions of HEK293 TRex FlpIN cells expressing doxycyclin-inducible shRNA targeting PCNA and simultaneously expressing eGFP-PCNA_WT. The two lower blots were incubated with an anti-PCNA antibody. B) Western Blot analysis of HEK293 TRex FlpIN shPCNA + eGFP-PCNA_WT chromatin extracts (anti-PCNA antibody). C&D) Western Blot analysis as described in A&B with HEK293 TRex FlpIN cells expressing shPCNA and the eGFP-PCNA_K164R mutant.

ATAD5 is not directly involved in DNA replication

We reasoned that ATAD5 could be involved in DNA replication due to its physical association with the replication fork *via* PCNA and its possible role as a PCNA unloader [18]. We therefore analyzed replication patterns and origin firing using the DNA fibre technique in two different A2780 clones that express distinct shRNAs targeting ATAD5 (clones 1-6 and 15-3) upon addition of doxycycline to the cell culture medium. The length of CldU tracks was unaffected upon ATAD5 downregulation, demonstrating that replication fork speed is not dependent on ATAD5 status under control conditions (Fig. 5 B&C). Furthermore, a quantification of the relative abundance of certain DNA fibre structures (displayed in Fig. 5 D) showed that there was no significant difference in the percentage of stalled replication forks or late origins fired (Fig. 5 E). Collectively, these results indicate that ATAD5 is not directly involved in replication under native conditions, despite its direct association with the replication fork mediated by its PCNA interacting motif (PIP). Additional proof for this was provided by another set of experiments where we overexpressed eGFP-tagged wild type ATAD5 in HEK293 TRex FlpIN cells. Tow different clones expressing ectopic eGFP_ATAD5 (clones 1.2 and 2.4) upon doxycycline supplementation showed no apparent replication fork progression defects (Fig. 5 F&G). ATAD5 overexpression alone thus does not appear to lead to uncontrolled unloading of PCNA from chromatin, which would interfere with active replication.

In order to test whether ATAD5 may instead be required for replication of damaged DNA, we also measured replication fork speed in ATAD5-depleted cells that have been exposed to different DNA damaging agents previously shown to induce hypersensitivity in those cells (Fig. 1). None of these agents (MNNG, MMC or Olaparib) had any significant impact on replication fork rates at the indicated exposure times and concentrations (Fig. 6 A-C). We therefore conclude that ATAD5 is indeed not directly involved in replication, neither on undamaged DNA nor across different types of lesions induced by MNNG, MMC or Olaparib. Alternatively, it may be required, like the yeast Elg1/RFC2-5 complex, for PCNA unloading after replication and ligation of each Okazaki fragment had been carried out [15]. These results further suggest that there might be an alternative way to 'recycle' PCNA, in order to provide a sufficient pool of free PCNA that can be loaded again at each Okazaki fragment to facilitate normal replication. One may envision that alternative unloader complexes take over when ATAD5 is missing, or that the PCNA trimer may even spontaneously open and disassociate.

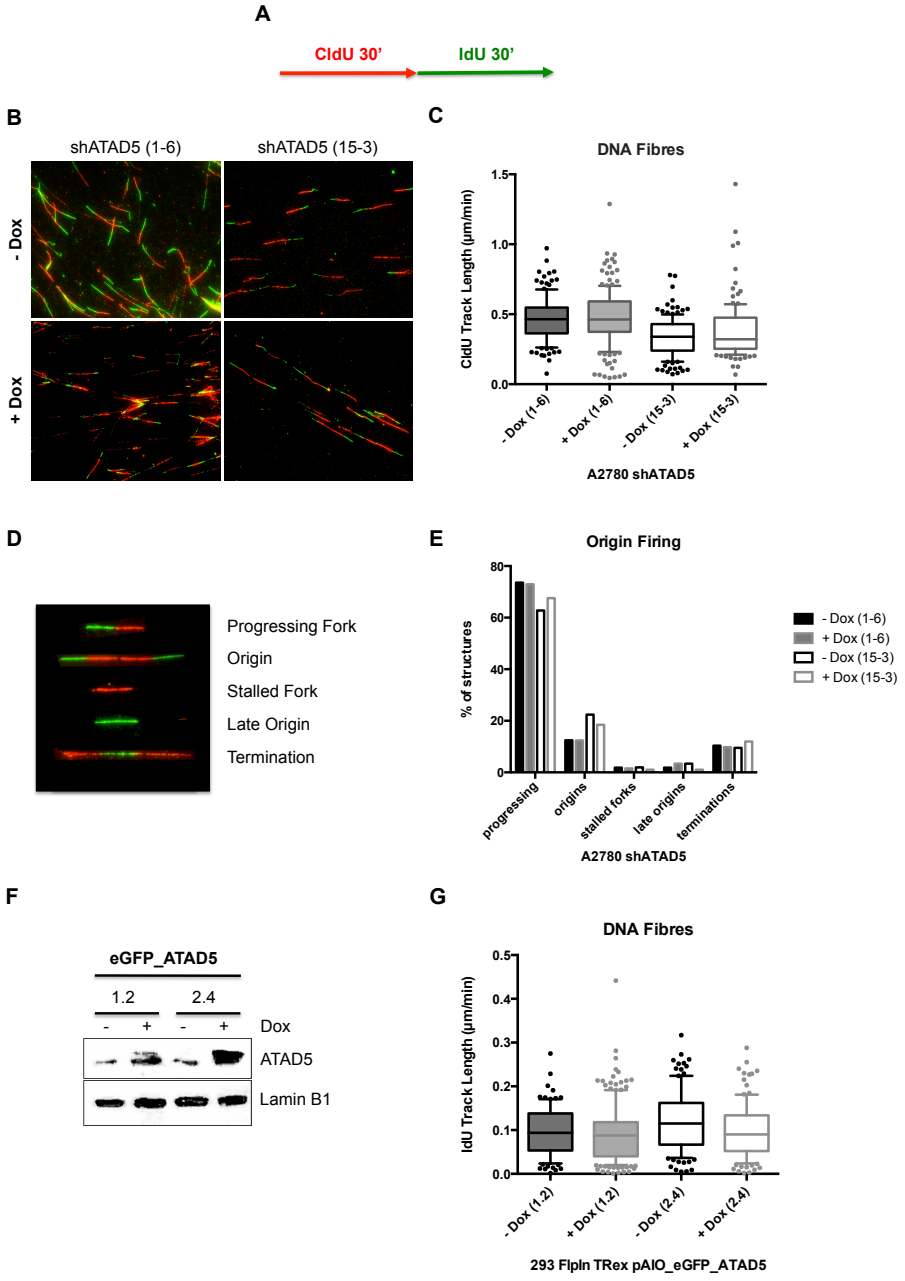

Fig.5 A) Schematic representation of the labeling protocol with CldU and IdU in a DNA fibre assay. Cells cultured in the absence or presence of 100 ng/ml doxycycline (Dox) were supplemented first with CldU, washed once and subsequently exposed to IdU in the cell culture medium. B) Representative images of A2780 shATAD5 (1-6 and 15-3) cells labeled with CldU and IdU for 30min each. C) Quantification of replication fork speed (represented as CldU track length) of indicated clones. Only progressing replication forks (see D) were analyzed. D) Representative images of individual DNA fibre structures. E) Quantification of relative amount of before-mentioned DNA fibre structures in A2780 shATAD5 (1-6 and 15-3) clones. F) Western Blot of doxycycline-inducible expression of stably transfected eGFP_ATAD5 in HEK293 TRex FlpIN cells. Two clones (1.2 and 2.4) were chosen for analysis. G) Quantification of replication fork speed (represented as IdU track length).

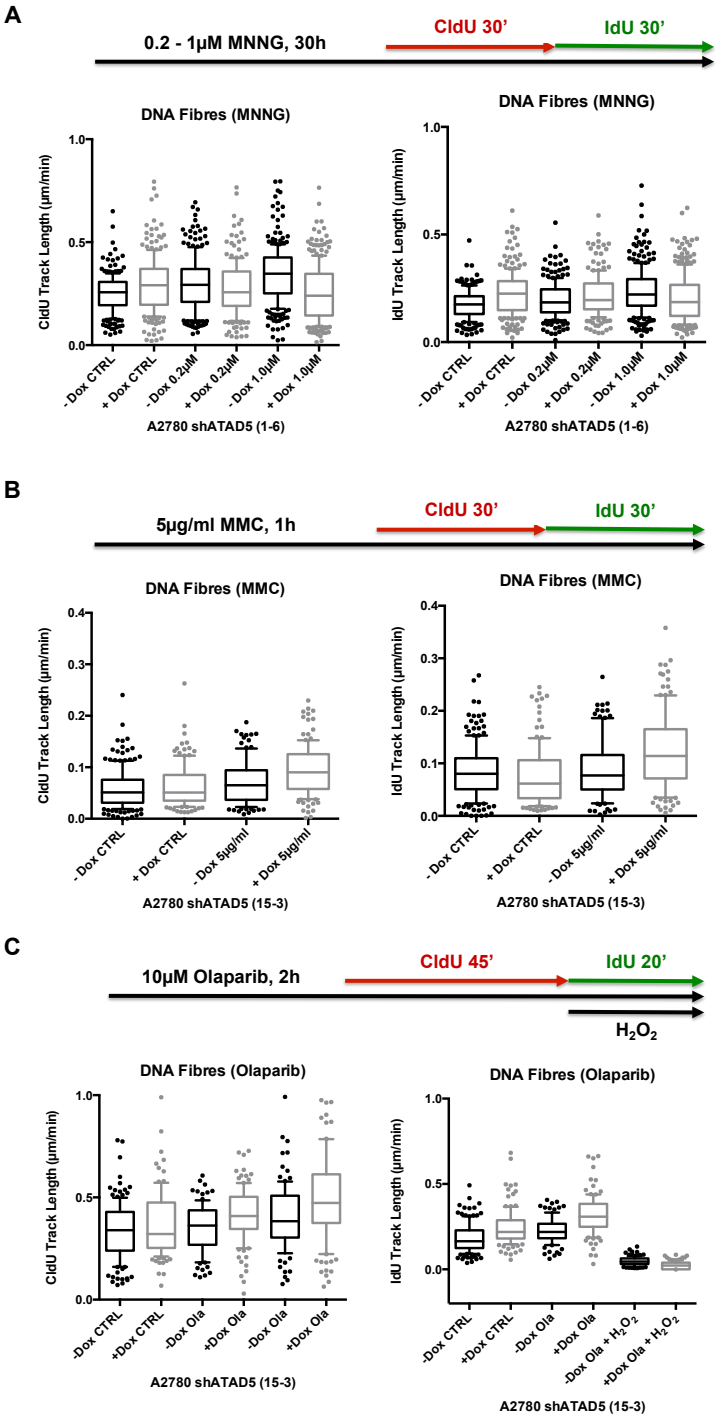

Fig.6 A) DNA fibre experiments performed using A2780 shATAD5 (1-6) cells upon pre-treatment with O^6-BG for 1h and exposure to MNNG at the indicated concentrations for 30h. The cells were subsequently labeled with CldU and IdU for 30min each and replication fork speed measured for both CldU and IdU tracks. B) DNA fibre experiments in A2780 shATAD5 (15-3) cells upon exposure to MMC at 5 μM/ml for 1h and subsequent labeling with CldU and IdU for 30min each. C) DNA fibre experiments using A2780 shATAD5 (15-3) cells treated with 10 μM Olaparib for 2h before exposure to CldU for 45min and IdU for 20min.

Discussion

ATAD5 has been suggested to play an important role in maintaining genome stability. We set out to investigate the exact function and molecular mechanism of ATAD5 action in human cells with special focus on its predicted role as a PCNA unloader. Our results confirm a contribution of ATAD5 to preserving genome stability, especially upon exposure to DNA damaging agents, such as MNNG, MMC or Olaparib. This is highlighted by the fact that ATAD5-deficient cells are hypersensitive towards these agents and display increased accumulation of DNA DSBs and a greater propensity to apoptosis. While the hypersensitivity observed with MMC and Olaparib might point towards a role of ATAD5 in homologous recombination, previous experiments using DSB-reporter assays did not show a clear effect and varied depending on the siRNA sequence used to knock-down ATAD5. Controversial reports are also found in the literature on the role of ATAD5 in HR, some stating that Elg1-deficiency causes increased spontaneous recombination events, while others show reduced HR frequency in reporter assays [18, 19]. This could be due to a general difference in spontaneous recombination versus DSB-induced recombination events; however, since our results are also inconclusive, we cannot state at this point that ATAD5 indeed plays a role in HR of DSBs. One may speculate though that ATAD5 is involved in HR-mediated repair of stalled or collapsed replication forks, which are induced by MMC and Olaparib, respectively. We have not directly tested this hypothesis, but several of our experiments indicate that ATAD5 is not involved directly in the process of replication. First, we did not observe any significant sensitivity upon ATAD5-depletion towards replication inhibitors, such as camptothecin and hydroxyurea or the G4-quadruplex stabilizers TMPyP4 and S2T1-6OTD, all of which cause replication fork stalling. Furthermore, DNA fibre experiments provided more direct evidence, showing that replication fork speed was unaffected in ATAD5-deficient cells, both under control conditions and upon DNA damage induced by either MNNG, MMC or Olaprib. We also did not observe increased replication fork stalling or excessive usage of late origins under any of the above-mentioned conditions, indicating that there are no major problems causing replication blocks or deregulated origin firing. Additionally, overexpression of ATAD5 did not result in replication defects, suggesting that overrepresentation of ATAD5 alone does not lead to uncontrolled unloading of PCNA from the DNA, which would likely cause replication inhibition. This points towards a highly regulated role of ATAD5 in removing PCNA from DNA when replication (of individual Okazaki fragments) has finished, or alternatively, at specific sites in the genome. In agreement with these results, we found no major defect during cell cycle progression, except a slightly retarded progression through S-phase when ATAD5 was missing. It thus seems like replication fork progression and cell cycle progression are largely unaffected by ATAD5 status, despite the fact that unmodified, as well as ubiquitylated, PCNA accumulates on chromatin upon ATAD5 depletion. We also did not observe an enhanced DNA damage response (DDR) under normal conditions, as indicated by γH2AX immunofluorescence staining. Conversely, DNA strand breaks clearly accumulate upon exposure to DNA damaging agents, as discussed above. One may thus speculate that unloading of PCNA at sites of DNA damage or blocked replication forks is required to maintain genome stability, rather than unloading of PCNA from undamaged DNA. Alternatively, ATAD5 could be specifically required for removal of ubiquitylated PCNA, for instance to avoid translesion synthesis at stalled forks and promote other DNA repair pathways. In order to investigate whether accumulation of PCNA or $PCNA^{ub}$ is causing genomic instability upon ATAD5 depletion, we generated cell lines that enable downregulation of endogenous PCNA by shRNA and simultaneous overexpression of wild type PCNA or a mutant that cannot be ubiquitylated on lysine 164 (PCNA_K164R) any more. These cells will be useful tools to investigate whether the phenotypes observed upon ATAD5-depletion are dependent on

PCNA ubiquitylation or not. However, additional transfection of siRNA targeting PCNA will be required, due to the incomplete knock-down of the endogenous protein.
In order to characterize functional domains of ATAD5, we have generated cell lines that enable inducible replacement of endogenous ATAD5 with GFP-tagged wild type ATAD5 or different mutants (data not shown). Expressing ATPase-dead mutants (ATAD5_KR) will clarify whether ATP hydrolysis is required for PCNA unloading or other unknown functions of ATAD5. We have also generated a mutant that is unable to interact with PCNA *via* its PCNA-interacting peptide (PIP) motif and another one, which cannot interact with the USP1-UAF1 complex that deubiquitylates $PCNA^{ub}$. Careful characterization of these mutants will hopefully shed light on the role of ATAD5 in preserving genome stability and domains required therefore.
In conclusion, this study has provided further evidence that mammalian ATAD5 is indeed involved in maintaining genome stability. However, thus far we were unable to characterize its exact function in DNA metabolism. This is mainly due to the difficulties to identify a pronounced and reproducible phenotype. We believe that ATAD5 is not directly involved in DNA replication, but unfortunately we cannot conclude thus far whether unloading of PCNA or $PCNA^{ub}$ is an essential function of ATAD5. Further experiments, using the generated tools, will hopefully clarify how ATAD5 operates to maintain genome stability.

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DISCUSSION

CANCER & DNA REPAIR

Genome instability is a hallmark of cancer and has been described as both a cause and a consequence of tumor initiation and progression [10, 11]. It is thus not surprising that novel approaches in oncology aim at targeting DNA damage signaling and repair pathways in an attempt to eradicate cancerous cells [7, 349]. In fact, ionizing radiation as well as commonly used chemotherapeutics, which have been applied for the treatment of human malignancies since many decades (*e.g.* nitrogen mustard analogues, antimetabolites, cisplatin and mitomycin C), induce DNA damage to inhibit tumor growth. This rationale has proven to be effective, because DNA lesions interfere with normal replication and transcription and even induce apoptosis when they exceed a certain threshold. DNA damage is thus a double-edged sword: on the one hand, it can drive and enhance tumor progression through enabling the evolution of cells with a proliferative advantage and resistance to anti-growth signals or other adverse conditions. On the other hand, generation of excessive DNA damage is used in the clinic to overload the capacity of cellular DNA repair mechanisms and trigger cell death by apoptosis. In general, chemotherapeutic agents that induce DNA damage are not very selective for cancer cells, but target any dividing cell or are more toxic to cells that replicate actively. Hence, administration of such drugs leads to side effects that are often quite severe and therefore dose-limiting. They are characterized by problems of the hematopoietic and digestive systems, the skin and hair follicles due to the high turnover and associated replication and growth rates of cells in these tissues.

A major focus of research in the past decade has therefore been put on the discovery and development of novel therapies that specifically target malignant cells and spare normal tissues. Interestingly, cancer cells themselves harbor high loads of DNA lesions due to oncogene activation, the microenvironment, or oxidative stress caused by aberrant metabolism [6, 350, 351]. Those cells are thus likely more dependent on active DNA repair mechanisms and inhibition of cell cycle regulators, such as p53, CHK1/2 and CDKs. Some tumors actually upregulate certain DNA repair pathways in order to increase their repair capacity and cope with the elevated levels of DNA damage; this was shown to induce resistance towards conventional chemotherapy [352-355]. Inhibition of DNA repair thus represents an interesting strategy to improve the efficacy of current anticancer treatment options and reduce the toxicity to normal cells. Some cancer cells exhibit defects in DNA repair, which results in replication stress that further drives genomic instability and thereby promotes tumorigenesis [356]. In this case, inhibition of a redundant or backup pathway mediates synthetic lethality, as cancer cells more heavily rely on those alternative repair mechanisms than normal cells. Numerous inhibitors targeting various different enzymes involved in DNA damage signaling and repair have already been generated, however, thus far only few have made it to the clinic.

One class of drugs targeting DNA repair proteins are PARP inhibitors, which specifically kill homologous recombination (HR)-deficient cancer cells as a consequence of a synthetic lethal interaction [2, 3]. PARP1 is an enzyme involved in the repair of single-strand breaks (SSBs), lesions that must be repaired rapidly, before the replication machinery encounters them, in order to avoid their collapse into deleterious double-strand breaks (DSBs). Efficient and faithful repair of DSBs requires an active HR pathway, which uses the intact sister chromatid for repair. While in normal cells both the SSB repair pathway, as well as the HR repair pathway are intact, BRCA1- or BRCA2-mutated cancer cells lack functional HR and are thus sensitive to inhibition of PARP, due to an accumulation of toxic DNA strand breaks.

Small molecule PARP inhibitors are actually used as stand-alone monotherapies for the treatment of BRCA1- or BRCA2-mutated cancers. In an intact genome where strand breaks arise rarely, HR can deal with their repair without having to rely on PARP, so its activity should be dispensable in cells that have not been challenged with exogenous DNA damaging agents. Due to loss of heterozygosity of either BRCA1 or BRCA2, HR is inactivated in tumor cells of patients. Furthermore, as a consequence of enhanced metabolism and oncogene activation, cancer cells do exhibit high levels of endogenous DNA damage in comparison to normal cells. We thus hypothesized that the underlying sources of DNA lesions leading to PARP inhibitor sensitivity are a consequence of endogenous stress that causes accumulation of SSBs in the genomic DNA. The exact type of DNA lesion contributing to PARP inhibitor sensitivity in HR-deficient cells has been a matter of speculation and not proven experimentally thus far.

One very common source of DNA damage is oxidation. Reactive oxygen species (ROS) are generated for instance due to oncogene activation and as byproducts of metabolism that is elevated in cancer cells. Especially the bases in the DNA are highly susceptible to oxidation, which can change their base-pairing properties; 8-oxoguanine (G^O) for instance can base-pair with both C and A. G^O:A mispairs are potentially mutagenic (when G^O is in the template strand) and their repair is initiated by the base-excision repair (BER) glycosylase MYH that excises the misincorporated A to allow insertion of a correct C [255]. MYH-induced repair of G^O:A base-pairs thus creates transient SSBs as repair intermediates that could activate PARP to facilitate efficient downstream repair.

Oxidative DNA damage repair initiated by MYH contributes to PARP inhibitor sensitivity in HR-deficient cells

We hypothesized that oxidative lesions generated by endogenous ROS are contributing to PARP inhibitor sensitivity in HR-defective cancer cells. To test this we co-depleted MYH together with BRCA1 in several different cancer cell lines and found that knock-down of MYH reduces the toxicity of the PARP inhibitor Olaparib in these cells. Furthermore, SSB and DSB accumulation was reduced when cells were depleted of MYH, indicating that processing of G^O:A mispairs by MYH actually does lead to genomic instability and

consequently sensitivity to treatment with Olaparib. Similar results were obtained upon depletion of MYH together with Rad51, suggesting that this is a general effect, which is true for HR-deficient cells and not dependent on BRCA1 status alone. In addition, these experiments prove that intrinsic oxidative stress substantially contributes to PARP inhibitor sensitivity and that PARP is indeed involved in the processing of MYH-mediated BER *in vivo*.

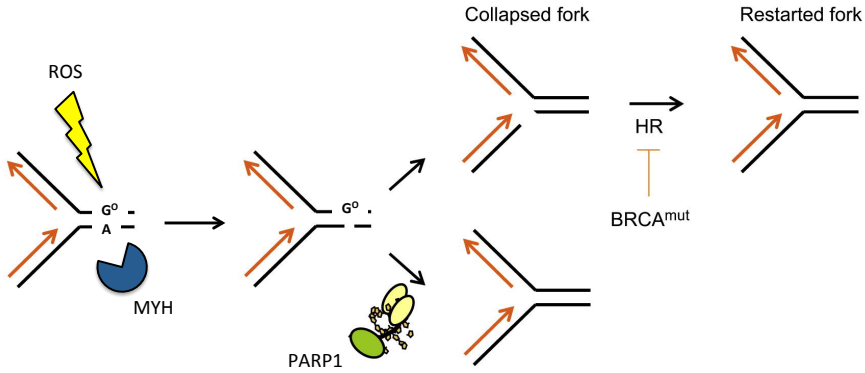

Fig.22: Model for MYH-induced BER of oxidative DNA damage leading to PARP inhibitor toxicity in HR-deficient cells. A) G^o/A mispairs are addressed by MYH, generating transient SSBs in the genome that may require PARP1 activity for repair, which is especially important in an HR-defective background (modified from Helleday, 2011 [326]).

These results may have some interesting clinical implications, as one may envision that combination of PARP inhibitors with agents that induce oxidative DNA lesions, such as Methotrexate, could potentiate their effect in the described genetic background. Methotrexate is already used in oncology to treat various types of cancer and has actually been shown to induce 8-oxo-guanine lesions in the DNA. With regards to personalized medicine, MYH status could serve as a useful biomarker to predict the efficacy of PARP inhibitors. According to our results, we would predict that reduced expression, loss, or mutation of MYH induces (acquired) resistance to Olaparib and other PARP inhibitors. Preliminary evidence (KuDOS) indeed suggests that MYH levels are low in cells that are resistant to PARP inhibitors. However, large-scale expression profiles of tumor samples of Olaparib responders and non-responders are required to confirm whether MYH status is of consequence for PARP inhibitor efficacy.

Our results are in line with previous reports stating that MYH induces lethal SSBs in the nucleus as well as mitochondria under conditions of oxidative stress [357]. These breaks eventually trigger two different cell death pathways in order to avoid mutagenesis. It is likely that under high load of oxidative stress, MYH-mediated BER creates an exceedingly high number of transient SSBs that activate PARP. Consequently, PARP catalyzes excessive poly-ADP-ribosylation, leading to rapid depletion of the cellular NAD^+ and ATP storage and ultimately causing cell death. This scenario probably does not apply under normal conditions, when the amount of DNA damage is low and merely due to endogenous

oxidative or metabolic stress. However, it shows that SSBs generated as transient intermediates during BER have the potential to activate PARP and induce cell death.

Considering this, one may speculate that next to MYH also other repair pathways might generate transient SSB repair intermediates that could be 'highjacked' by PARP and thus induce PARP inhibitor sensitivity in an HR-deficient background. With respect to oxidative DNA damage, OGG1 as well as the NEIL family members (NEIL1-3) come to mind. These BER glycosylases recognize and excise different types of oxidized nucleotides in the DNA and are therefore interesting candidates. Our preliminary results on OGG1 showed increased DSB accumulation and a high variability in the survival experiments (data not shown). Furthermore, we observed a surprising toxicity of OGG1 knock-down alone, which could mask any effect on resistance and genomic instability induced by PARP inhibitors in OGG1-depleted cells. Interestingly, the mechanism of action of MYH- and OGG1-mediated BER is different. While MYH is a DNA glycosylase that is thought to induce long-patch BER, which requires the activity of PCNA, RPA and Polδ/ε, as well as FEN1 and LigI, OGG1 is a glycosylase/lyase that triggers short-patch BER, carried out by Polβ, XRCC1 and LigIII. The process of long-patch BER presumably requires more time than the short-patch repair process, which could allow sufficient time for the recruitment of PARP specifically to long-patch repair sites, where it may be required to coordinate the repair process. Additionally, MYH-initiated repair of G^{O}:A mispairs restores G^{O}:C pairs that additionally need OGG1 activity to restore the original G:C. This two-step repair process is consequently much more time consuming and requires the induction of at least two transient SSBs before the G:C base pair can be re-established. Another important difference is that, in contrast to MYH, OGG1 excises G^{O} from lesions that are not directly mutagenic, namely, G^{O}:C pairs. These lesions can arise throughout the cell cycle by oxidation of G residues in genomic DNA. G^{O}:A mispairs (with G^{O} in the template strand) on the other hand, which are recognized by MYH, can only be generated during S-phase by misincorporation of A opposite an already oxidized G^{O} present in the template strand. This is why MYH activity must be high during S-phase to efficiently take care of those lesions, while OGG1 has sufficient time to repair G^{O}:C pairs throughout all phases of the cell cycle. With regards to PARP inhibitor sensitivity, it would be very interesting to dissect the differential roles of MYH and OGG1 in the repair of oxidative DNA lesions and their impact on PARP activation, as this might have important implications for personalized medicine.

Due to time constraints, we were not able to investigate in detail the contribution of other BER glycosylases, such as the NEILs, UNG, SMUG1, TDG, MBD4, MPG, NTH1 on PARP inhibitor sensitivity in HR-deficient cells. However, it is likely that next to oxidative DNA damage, processing of other types of endogenous DNA lesions can trigger PARP activity through the generation of transient repair intermediates. Spontaneous depurinations also represent a threat to genome integrity, leading to loss of about 5000 purines per cell per day. The resulting apurinic sites are recognized and incised by APE1, generating more potential substrates for PARP. Furthermore, it is believed today that APE1 is required during most, if not all, BER reactions for incision of abasic sites or at least for trimming of

inappropriate ends generated by bifunctional glycosylases that are not ready for gap filling by the polymerases. Inhibition of APE1 could therefore potentially have the greatest impact amongst BER enzymes on the contribution to PARP inhibitor sensitivity in HR-deficient cells. However, APE1 is an essential enzyme, which is why its depletion or inhibition is highly cytotoxic and might mask the effects that we aimed at investigating. There are also other end-processing enzymes, such as PNKP, which are required to create suitable DNA ends following DNA glycosylase-mediated excision of the base. These are clearly interesting candidates, since it is also possible that such enzymes could prevent PARP activation, instead of promoting it, by binding with high affinity to the SSBs and thereby abolish access of PARP to the nicks. However, this remains a matter of speculation and will be clarified by future investigations.

Enzymes that remove damaged nucleotides from the DNA precursor pool could potentially also contribute to PARP inhibitor sensitivity. MTH1 is a hydrolase that degrades oxidized nucleotides, such as 8-oxo-dGTP and 2-OH-dATP, to prevent their incorporation into the DNA during replication. Recently, it was shown that inhibitors of MTH1 induce 'cancer phenotypic lethality' [8, 287]. MTH1 is thus considered to be an essential protein for cancer cells, while being dispensable in normal cells where levels of oxidative stress are low. MTH1 inhibitors increase the levels of oxidative DNA damage due to enhanced incorporation of oxidized nucleotides, which should then be substrates for MYH- or OGG1-induced BER that create transient SSBs. A combination therapy using both PARP and MTH1 inhibitors in a HR-deficient background may represent a promising strategy. We have tested this hypothesis experimentally (data not shown) and found that it is rather difficult to combine the MTH1 inhibitor TH588 with other drugs, due to the limited concentration window in which TH588 is active, but not yet toxic to the cells. However, preliminary experiments using siRNA to mediate knock-down of MTH1 together with BRCA1 point towards a small increase in Olaparib sensitivity when compared to BRCA1 knock-down alone (data not shown). Novel MTH1 inhibitors may be more useful to test the above-stated hypothesis and clarify whether MYH- or OGG1-initiated BER are capable of dealing with the increasing amount of oxidized nucleotides incorporated into the DNA in the absence of MTH1.

The experiments we have performed in this study were all carried out without addition of exogenous DNA damaging agents in order to mimic situations where endogenous stress is the predominant source responsible for the generation of DNA lesions. We expect, however, that addition of oxidizing agents, such as H_2O_2 or $KBrO_3$, would further increase the sensitivity of BRCA1-depleted cells towards PARP inhibitors. Conversely, co-depletion of MYH should reduce this toxicity, as it would prevent processing of oxidative lesions and thus avoid generation of PARP-activating intermediates. Preliminary results (data not shown) using H_2O_2 are difficult to interpret due to the labile nature of this compound and the resulting variability within different experiments. Nevertheless, it seems that H_2O_2 does have a negative effect on survival of BRCA1-depleted cells exposed to Olaparib. Notably, H_2O_2 induces at high concentrations not only oxidized DNA bases, such as

8-oxoguanine, but also DNA strand breaks and of course oxidation damage to proteins as well as lipids. This reduced specificity is especially important when considering results of other studies that apply high doses of H_2O_2 or other DNA damaging agents. The standard cell culture conditions also do not fully represent normal physiological conditions, especially the heterogenic tumor environment, where most cells are actually under hypoxic conditions, while others located in close proximity to the afferent vessels are actually highly oxygenated. Other factors in the microenvironment might also affect oxidative stress, especially tumor-infiltrating immune cells, which elicit a response that includes ROS production and release. Cell culture experiments are thus not able to fully recapitulate the complex nature of the tumor environment, especially with respect to factors that induce oxidative stress in a real tumor. On the other hand, it is also difficult to predict the amount of endogenous oxidative stress present in tumor cells and it might vary considerably between different cell types and even within the heterogenic population of tumor cells themselves.

Interestingly, we and others show that siRNA-mediated depletion of PARP1 dramatically reduces the toxicity of PARP inhibitors in BRCA1-deficient background [358], irrespective of MYH status. These results confirm the hypothesis that PARP inhibitors trap PARP on the DNA, as the enzyme cannot catalyze the addition of negatively-charged poly-ADP-ribose polymers on itself that would normally lead to repulsion of the protein from the DNA. The resulting PARP-DNA complexes are highly cytotoxic, presumably representing major blocks to replication and transcription [358]. Furthermore, they require repair mediated by HR, which is impeded in BRCA1-depleted cells. This would also explain the high amount of DNA DSBs in those cells upon treatment with Olaparib. In order to better understand this mechanism, one should use inhibitors of PARP, which block the DNA binding domains instead of the catalytic core. Furthermore, it would be interesting to dissect the contribution of other PARP family members involved in DNA repair, namely PARP2 and PARP3. Today we still rely on siRNAs to do so, because none of the currently available PARP inhibitors are strictly selective and target several different PARP family members.

As mentioned earlier, the sources of endogenous DNA lesions are manifold and do not only include oxidative DNA damage. We decided to investigate one other very common lesion, namely ribonucleotides (rNTPs), that are very frequently incorporated by the replicative polymerases. Ribonucleotides present in genomic DNA constitute a major threat to the stability of the genome and are normally repaired by RNaseH2 [359]. Our results indicate that despite the abundance of these lesions, RNaseH2-mediated repair of rNTPs does not contribute to PARP inhibitor sensitivity in HR-defective cells. In other words, processing of rNTPs does not create suitable SSB repair intermediates that require PARP activity. In contrast to MYH, RNaseH2-induced repair might either create repair

intermediates that are not substrates for PARP or not accessible to it due to tight binding of RNaseH2 or downstream repair factors. We actually observed increased levels of DSBs upon RNaseH2 depletion and treatment with PAPR inhibitors, confirming that, in the absence of RNaseH2, alternative processing or hydrolysis of rNTPs leads to an accumulation of strand breaks [359]. In order to confirm that RNaseH2-induced repair of these lesions really does not give rise to suitable substrates for PARP, *in vitro* assays should be carried out.

To quantify the contributions of different repair factors, it would be advantageous to base future work on a clean genetic system that does not rely on siRNA-mediated knock-down efficiencies or other variables that are difficult to control. BRCA1- or BRCA2 knock-out cell lines from patients do exist, however, they often have unstable phenotypes and are thus difficult to compare over time in cell culture setting. CRISPR/Cas9-mediated knock-out of HR-factors BRCA1 or BRCA2 in combination with MYH (or other glycosylases and repair enzymes) knock-out or depletion could be an interesting alternative to test our hypothesis in the future.

The role of ATAD5 in genome maintenance

Thus far, PARP inhibitor toxicity has been reported in cells displaying a defect in homologous recombination, due to the synthetic lethal interaction of this DNA repair pathway with SSB repair. Surprisingly, we found that cells depleted of ATAD5 are also hypersensitive to treatment with Olaparib. A role for ATAD5 in genome stability has already been suggested a few years ago [61]; however, the observation that ATAD5-deficient cells are particularly sensitive to Olaparib is new and points towards a contribution of the protein in HR. Unfortunately, previous results using HR-reporter assays (DR-GFP) failed to give a clear answer to the question whether ATAD5 is indeed involved in the process of HR. It would therefore be interesting to further study HR in ATAD5-depleted cells, *e.g.* investigate the spontaneous cellular HR frequency by measuring sister chromatid exchange (SCE) rates, instead of quantifying the repair efficiency of an artificially induced double-strand break. We reasoned that instead of being required for HR at two-ended DSBs, ATAD5 function might instead be necessary for HR at collapsed replication forks that manifest as one-ended DSBs. We therefore carried out DNA fibre experiments that allow visualization of DNA replication tracks and therefore quantification of replication speed, origin firing and fork stalling. Similarly to results from the Myung lab [50], we did not observe any discrepancies in replication fork progression rates upon ATAD5 depletion in unperturbed cells. Additionally, we did not detect any increase in the percentage of late origin firing or early terminations. These results imply that origin firing is not deregulated and further show that downregulation of ATAD5 by itself does not lead to replication fork stalling or collapse on undamaged genomic DNA. In order to test whether ATAD5 may instead be important for replication over different DNA lesions, we exposed cells to DNA damaging

agents prior to DNA fibre analysis. Our results indicate that ATAD5 status does not affect replication of damaged DNA either, as fork speed was unperturbed upon exposure to MNNG, MMC and Olaparib, which all induce different types of DNA lesions. However, we have to take into consideration that although DNA fibre assays are useful tools to investigate global replication patterns, the technique is not very sensitive and relies on effects that are strong enough to affect replication on a genome-wide level. In other words, if the damage induced by certain agents is rare or only present in a minor fraction of the genomic DNA, it will not be detectable using DNA fibre analysis. It is thus easy to miss certain effects due to low drug concentration or specificity of lesions induced only, or preferentially, in certain regions of the genome. We cannot strictly rule out the possibility that this is the case here, however, we have chosen well studied agents and concentrations that are well above the toxic threshold and negatively affect survival of treated cells, while still allowing replication to take place. Interestingly, it was recently shown that lesions, such as interstrand crosslinks (ICLs), which were previously believed to cause strict replication fork blocks, are traversed to prevent stalling and collapse of active replication machineries [360]. It is thus reasonable to assume that also other types of DNA damage can be traversed and do not cause fork stalling, at least not for long periods of time. If this is indeed the case, DNA fibre assays would fail to detect them and thus underestimate the number of lesions generated by the applied DNA damaging agent(s). Such lesions could subsequently be repaired post-replicatively and we cannot exclude the possibility that ATAD5 is taking part in the later repair processes.

Nevertheless, ATAD5 travels with the replication machinery and has been implicated in the unloading of PCNA, as well as ubiquitylated and SUMOylated PCNA, from chromatin [49, 50]. Until recently, it was unclear whether ATAD5 is involved in global PCNA unloading, or whether removal of PCNA by ATAD5 is restricted to certain regions of the genome, such as centromeres, telomeres or sites of cohesion or DNA damage. New data, using yeast as a model system, showed that Elg1 (the yeast homolog of ATAD5) is required for PCNA unloading following Okazaki fragment ligation on a genome-wide scale [59]. However, it is unclear whether this is also true for human ATAD5. Furthermore, it also still remains enigmatic whether unloading of PCNA or rather its modified forms $PCNA^{ub}$ or $PCNA^{SUMO}$ is crucial to maintain genome stability. In an attempt to better understand the importance of the modification of PCNA regarding ATAD5s function, we have generated HEK293 TRex FlpIN cells that enable simultaneous downregulation of endogenous PCNA, while overexpressing eGFP-tagged wild type PCNA or a mutant, which cannot be modified on lysine 164 (K164R). This residue is normally ubiquitylated in response to replication stress in order to recruit translesion synthesis (TLS) polymerases to sites of DNA damage [31]. K164 is SUMOylated in S-phase, presumably inhibiting inappropriate recombination events by recruitment of anti-recombinases, such as Srs2 in yeast or PARI in human cells [35]. Unfortunately, due to time constraints, I was not able to test whether the phenotypes observed upon ATAD5 depletion are dependent on modification of PCNA or not. Nevertheless, the above-mentioned cell lines will hopefully serve as useful tools to test this

experimentally. I would like to mention at this point that it has proven rather difficult to characterize ATAD5-deficient cells phenotypically, due to effects on genome stability that are minor at best and a high variability within the individual experiments. This could be due to redundant functions of ATAD5 with other proteins, such as alternative PCNA unloaders, or because ATAD5 plays only a minor role in genome maintenance in human cells. Furthermore, discrepancies and contradictions within the literature have aggravated research concerning ATAD5. Despite these difficulties, it remains a highly interesting candidate in the DNA repair field and deserves to be investigated further. If we manage to find conditions, where ATAD5 is absolutely required to maintain low levels of DNA damage that are compatible with cell survival, we might be able to dissect its basic biological function.

Especially its proposed role as a PCNA unloader is interesting, since PCNA is a central player in DNA metabolism, orchestrating DNA replication and repair events [23]. We believe that ATAD5 is not directly involved in replication, since depletion of ATAD5 did not cause any replication defects as mentioned earlier. Neither did ATAD5 overexpression, which shows that removal of PCNA from chromatin by ATAD5 is a highly regulated process that likely requires completion of Okazaki fragment ligation (as in yeast) or another type of signaling. Preliminary data from our laboratory indicate that ATAD5 may have a role in the timely repair of single-strand breaks induced by the oxidizing agent $KBrO_3$ (data not shown). These results are in line with the experimental evidence reported here, showing that ATAD5-depleted cells have reduced capacity to repair DNA lesions induced by MNNG, MMC or Olaparib, as measured using Pulse Field Gel Electrophoresis and the Alkaline Comet assay. Further experiments are required to confirm these observations and one aspect of our future work will surely focus the recovery of DNA strand breaks. SSBs are especially interesting in this context, because they are recognized by PARP and we show here that ATAD5-deficient cells are hypersensitive to treatment with the PARP inhibitor Olaparib. A combinatory treatment of Olaparib together with DNA strand-break inducing agents could therefore potentiate the effect of ATAD5-depletion and give rise to a more robust phenotype. Endogenous oxidative stress is one of the most common threats to genomic stability, causing oxidative DNA damage as well as DNA strand breaks that in turn activate PARP. ATAD5 may be required for the repair of such lesions, which would explain the sensitivity of ATAD5-deficient cells to PARP inhibitors and the delayed repair of $KBrO_3$-induced SSBs. With respect to this, it would be interesting to investigate Olaparib sensitivity upon ATAD5 depletion in an HR-deficient background. If ATAD5 is not directly involved in HR, it should potentiate PARP inhibitor sensitivity in those cells and thus not show an epistatic effect.

Thus far, we have not been able to fully uncover the role of ATAD5 in maintaining genome stability and elucidate whether removal of PCNA has an important impact in this respect. Future work with the generated tools will hopefully resolve these unanswered questions and give important insights into DNA metabolism connected to ATAD5 and PCNA.

CONCLUDING REMARKS & FUTURE PERSPECTIVES

Synthetic lethality has been proven to be a valid approach to selectively kill cancer cells, however, resistance often occurs and reduces the applicability of agents used in such therapeutic approaches. It is thus highly important to understand the basic biological mechanism and find suitable biomarkers in order to predict possible clinical outcome and enable personalized medicine. A synthetic lethal interaction between PARP inhibitors and homologous recombination (HR)-deficient cells was demonstrated about ten years ago experimentally and has recently been applied in the clinic [2, 3]. PARP inhibitors are currently used for treatment of BRCA1- and BRCA2-mutated breast, ovarian and prostate cancers. However, they represent only the fourth line treatment options thus far and several resistance mechanism have already been described [182, 346, 347], hampering the efficacy of these agents in certain patients. Surprisingly, the exact biological role of the main target, PARP1, is not fully unraveled and mechanistic insights into the molecular basics are also incomplete.

With this study we took an important step in clarifying the mode of action of PARP inhibitors in HR-deficient cells. By showing that MYH-depletion reduces Olaparib toxicity, we were able to demonstrate that endogenous oxidative DNA damage represents one underlying source of single-strand breaks, which eventually feed into the HR pathway inactivated by BRCA malfunction. These results further imply that hypoxia and low expression or mutation of MYH will negatively affect PARP inhibitor efficacy in those tumors. As far as personalized medicine is concerned, these novel insights might help to exclude patients, which will likely not benefit from treatment with these agents. Other sources of endogenous stress remain to be identified, however, we were able to show that RNaseH2-mediated processing of ribonucleotides in genomic DNA does not contribute to PARP inhibitor sensitivity. Furthermore, it is clear that alternative mechanisms, such as PARP trapping on DNA, must lead to the toxicity of PARP inhibitors, since PARP-depletion reduced the sensitivity of Olaparib. Taken together, these results show how important it is to gain a detailed understanding of the basic mechanism of clinically relevant chemotherapeutics and all biological pathways that are involved in order to design and develop more efficient treatment regiments.

Interestingly, we observed that ATAD5-deficient cells display hypersensitivity to PARP inhibitors. This would place ATAD5 in the HR pathway, according to the proposed synthetic lethality model. However, it has also been shown that PARP trapping on DNA is actually more toxic to cells than inhibition of the catalytic activity of PARP. We may thus speculate that ATAD5, as a proposed PCNA unloader, normally prevents PCNA trapping on DNA. It is unclear at the moment if and how PCNA that remains loaded on chromatin is toxic to cells,

but ATAD5-depletion, which enhances PCNA levels on DNA, clearly induces a genomic instability phenotype. Whether PCNA accumulation on chromatin upon ATAD5-depletion and genomic instability are connected is still under investigation and remains a highly interesting question to answer. Because PCNA is a platform for various DNA replication and repair factors, it orchestrates many of these events and is thus crucial for their timely and spatial regulation. It would also be interesting in this respect to study the exact roles of PCNA modifications, especially ubiquitylation and SUMOylation, as they are substantially involved in the regulation of replication and repair pathways. Since DNA replication is not affected by either downregulation or overexpression of ATAD5, we conclude that ATAD5 is not directly involved in replication, but might instead be required for PCNA unloading after Okazaki fragment maturation or alternatively in post-replicative repair processes.

To date, we have not been able to fully unravel the precise biological function of ATAD5. However, due to its proposed role as a PCNA unloader and gatekeeper of the genome it might have possible clinical implications, due to the hypersensitivity towards Olaparib and other DNA damaging agents. If it proves to be a factor involved in homologous recombination, it could broaden the spectrum of tumors treated with PARP inhibitors, in case ATAD5-deficiency is observed in any tumor types. We are far from such deeds, however, investigating the basic molecular mechanism of ATAD5 and its connection to PCNA will surely bring us one step closer to understanding important facets of global DNA metabolism.

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ACKNOWLEDGEMENTS

Professor Josef Jiricny - thank you for your steady support, guidance and supervision throughout my PhD. Your enthousiasm for science has always been encouraging, motivating and fascinating! You have also created a wonderful working atmosphere always kept an open ear and mind for us, which has eased most, if not all, our PhD troubles. I am deeply grateful for the many opportunities you have provided me with and will treasure what I have learned in the past three years!

My Committee Members - Professor Matthias Peter and Dr. Heinz Jacobs, thank you for your constructive criticism on both of my projects and keeping up with the changes throughout this thesis.

Julia, Lia and Martin - thank you for your incredible frindship and the many good times we spent together in and out of the lab! It was deeply comforting to share PhD frustrations and successes with you and also to enjoy the sunny side of life together. I hope we will stay connected as we are now, wherever life will lead us!

Anja, Simone, Svenja - thank you for your frindship and help in the lab! I have always looked up to you and hope you know that you have made an impact on me!

Antonio, Maite, Mariela, Medini, Rachel, Saho, Sara, Sascha, Shun - thank you for being part of a great lab! The lab wouldn't have been the same without any of you.

Danielle, Eveline, Bettina, Jana, Chris - danke, dass ihr unsere tausend Fragen erduldet und beantwortet und uns unterstützt, wo es nur geht!

Farah & Odete - thank you two for being a wonderful and unreplacable part of the IMCR. You make our lives so much easier every day and it's a pleasure to have you around!

IMCR - thank you guys lots of funny discussions in the labs and hallways! It's a pleasure to see your happy faces in the mornings.

Leiden - thanks for being a calculated risk that payed of in every way! It was an exciting challenge and prepared me for lots of wonderful lively adventures, ever since.

Stockholm - thanks Anna, Dennis, Philipp, Thore; Andreas, Bastiaan, Cissi, Helena, Mark, Oliver, Patrick, Petra, Sabin; Ann-Sofie, Helge, Niklas and Thomas for an amazing year up North. I have the most vivid and joyful memories and still miss you, Sweden!

Brigitte, Corinna, Sarah - danke für eure langjährige Freundschaft! Es ist immer eine Freude nach Hause zu kommen und zu wissen, dass ich euch dort finde.

Janina, Katha, Lea und Sarah - danke dass ihr mich bis heute durch alle meine Lebensstationen begleitet, egal wo es mich hin verschlägt. Ich hoffe das Leben bereitet uns noch einige gemeinsame Abenteuer!

Donna, Kaitie, Me-Mom & Pop-Pop - thanks for the amazing exchange year you made possible for me more than 10 years ago. Time flies and I cherish our continued deep affection. This experience has affected me, my life and my future in so many good ways, and it's been a wonderful ride, ever since!

Meine Familie: Mama & Papa, Julia & Lukas, Stefan, Omi & Tante, Opa & Oma - DANKE, dass ihr die seid, die ihr seid! Bei euch bin ich Zuhause, wo ich Liebe, Geborgenheit, Halt und Sicherheit finde. Danke, dass ihr mich immer für mich da seid, mich unterstützt, motiviert, anspornt und herausfordert!

Mama & Papa - DANKE! Ihr seid meine Felsen in der Brandung, mein Rückhalt und meine Ruhe! Zu wissen, dass ihr hinter mir steht macht es so viel leichter den Abenteuern und Herausforderungen des Lebens mit offenen Armen und strahlenden Augen entgegen zu treten!

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